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**UTILITY
PATENT APPLICATION
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(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. 7682-055-999 Total Pages 85

First Named Inventor or Application Identifier

Hong Jin

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PTC6867 U.S. PRO
11/28/00
09/72/379**APPLICATION ELEMENTS**
See MPEP chapter 600 concerning utility patent application contents.ADDRESS TO: Assistant Commissioner for
Box Patent Application
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Submit an original, and a duplicate for fee processing)2. ☒ Specification [Total Pages 68]
(preferred arrangement set forth below)

- Descriptive title of the Invention
- Cross Reference to Related Applications
- Statement Regarding Fed sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention

- Brief Description of the Drawings (if filed)
- Detailed Description of the Invention (including drawings, if filed)
- Claim(s)
- Abstract of the Disclosure

3. ☒ Drawing(s) (35 USC 113) [Total Sheets 12]4. ☐ Oath or Declaration [Total Sheets]

- a. ☐ Newly executed (original or copy)
- b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)

[Note Box 5 below]

i. ☐ **DELETION OF INVENTORS(S)**

Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33 (b).

- ☐ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)7. ☐ Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)

- a. ☐ Computer Readable Copy
- b. ☐ Paper Copy (identical to computer copy)
- c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

- 8. ☐ Assignment Papers (cover sheet & document(s))
- 9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)
- 10. ☐ English Translation Document (if applicable)
- 11. ☐ Information Disclosure ☐ Copies of IDS
Statement (IDS)/PTO-1449 Citations
- 12. ☐ Preliminary Amendment
- 13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
- 14. ☐ Small Entity ☐ Statement filed in prior application,
Statement(s) Status still proper and desired
- 15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)
- 16. ☐ Other:

17. If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:

- ☐ Continuation ☒ Divisional ☐ Continuation-in-part (CIP) of prior application No: 09/161,122 filed September 25, 1998.

18. CORRESPONDENCE ADDRESS☒ Customer Number or Bar Code Label

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Prior application: 09/161,122 Examiner B. BrumbackArt Unit 1642

Assistant Commissioner for Patents
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Sir:

This is a request for filing a ☒ continuation ☐ divisional application under 37 CFR § 1.53(b), of pending prior application no. 09/161,122, filed on September 25, 1998; which claims priority to prior application no. 60/089,207, filed June 12, 1998, prior application no. 60/084,133, filed May 1, 1998, and prior application no. 60/060,153, filed September 26, 1997.

of HONG JIN, PETER PALESE, and DAVID KIRKWOOD CLARKE
(inventor(s) currently of record in prior application)

for RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES
(title of invention)

1. ☒ The filing fee is calculated below:

PATENT APPLICATION FEE VALUE

TYPE	NO. FILED	LESS	EXTRA	EXTRA RATE	FEE
Total Claims	6	-20	0	\$18.00 each	\$ 0.00
Independent	2	-3	0	\$80.00 each	\$ 0.00
Basic Fee					\$ 710.00
Multiple Dependency Fee If Applicable (\$270.00)					\$ 0.00
Total					\$ 710.00
50% Reduction for Independent Inventor, Nonprofit Organization or Small Business Concern					- \$ 0.00
Total Filing Fee					\$ 710.00

2. ☒ Please charge the required fee to Pennie & Edmonds LLP Deposit Account No. 16-1150. A copy of this sheet is enclosed.
3. ☒ Amend the specification by inserting before the first line the following sentence: This is a division, of application no. 09/161,122 , filed September 25, 1998.

- 4a. ☐ Transfer the drawings from the prior application to this application and abandon the prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file.
- 4b. ☐ New formal drawings are enclosed.
- 4c. ☒ Informal drawings are enclosed.
- 5a. ☐ Priority of application no. filed on in is claimed under 35 U.S.C. §119.
- 5b. ☐ The certified copy has been filed in prior application no. , filed .
6. ☒ The prior application is assigned of record to AVIRON, INC..
- 7a. ☒ The Power of Attorney appears in the original papers in the prior application no. 09/161,122, filed September 25, 1998.
- 7b. ☐ Since the Power of Attorney does not appear in the original papers, a copy of the Power in prior application no. , filed is enclosed.
8. ☐ This application contains nucleic acid and/or amino acid sequences required to be disclosed in a Sequence Listing under 37 CFR §§1.821-1.825. It is requested that the Sequence Listing in computer readable form from prior application no., filed on be made a part of the present application as provided for by 37 C.F.R. §1.821(e). The sequences disclosed therein are the same as the sequences disclosed in this application. A copy of the paper Sequence Listing from application no. is enclosed.
9. ☐ The undersigned states, under 37 C.F.R. §1.821(f), that the content of the enclosed paper Sequence Listing from application no. is the same as the content of the computer readable form submitted in application no. .
10. ☐ Additional enclosures or instructions.

Respectfully submitted,

by: *Jaqueline Penn*
Reg No. 43,492

Date November 28, 2000

Laura A. Coruzzi

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RECOMBINANT RSV VIRUS EXPRESSION SYSTEMS AND VACCINES

1. INTRODUCTION

The present invention relates to recombinant
5 negative strand virus RNA templates which may be used to
express heterologous gene products in appropriate host cell
systems and/or to construct recombinant viruses that express,
package, and/or present the heterologous gene product. The
expression products and chimeric viruses may advantageously be
10 used in vaccine formulations. In particular, the present
invention relates to methods of generating recombinant
respiratory syncytial viruses and the use of these recombinant
viruses as expression vectors and vaccines. The invention is
described by way of examples in which recombinant respiratory
15 syncytial viral genomes are used to generate infectious viral
particles.

2. BACKGROUND OF THE INVENTION

A number of DNA viruses have been genetically
20 engineered to direct the expression of heterologous proteins
in host cell systems (e.g., vaccinia virus, baculovirus,
etc.). Recently, similar advances have been made with
positive-strand RNA viruses (e.g., poliovirus). The
expression products of these constructs, i.e., the
25 heterologous gene product or the chimeric virus which
expresses the heterologous gene product, are thought to be
potentially useful in vaccine formulations (either subunit or
whole virus vaccines). One drawback to the use of viruses
such as vaccinia for constructing recombinant or chimeric
30 viruses for use in vaccines is the lack of
variation in its major epitopes. This lack of variability in
the viral strains places strict limitations on the repeated
use of chimeric vaccinia, in that multiple vaccinations will
generate host-resistance to the strain so that the inoculated
35 virus cannot infect the host. Inoculation of a resistant
individual with chimeric vaccinia will, therefore, not induce
immune stimulation.

By contrast, negative-strand RNA viruses such as influenza virus and respiratory syncytial virus, demonstrate a wide variability of their major epitopes. Indeed, thousands of variants of influenza have been identified; each strain
5 evolving by antigenic drift. The negative-strand viruses such as influenza and respiratory syncytial virus would be attractive candidates for constructing chimeric viruses for use in vaccines because its genetic variability allows for the construction of a vast repertoire of vaccine formulations
10 which will stimulate immunity without risk of developing a tolerance.

2.1. RESPIRATORY SYNCYTIAL VIRUS

Virus families containing enveloped single-stranded
15 RNA of the negative-sense genome are classified into groups having non-segmented genomes (Paramyxoviridae, Rhabdoviridae) or those having segmented genomes (Orthomyxoviridae, Bunyaviridae and Arenaviridae). Paramyxoviridae have been classified into three genera: paramyxovirus (sendai virus,
20 parainfluenza viruses types 1-4, mumps, newcastle disease virus); morbillivirus (measles virus, canine distemper virus and rinderpest virus); and pneumovirus (respiratory syncytial virus and bovine respiratory syncytial virus).

Human respiratory syncytial virus (RSV) is the leading
25 cause of severe lower respiratory tract disease in infants and young children and is responsible for considerable morbidity and mortality. Two antigenically diverse RSV subgroups A and B are present in human populations. RSV is also recognized as an important agent of disease in immuno-compromised adults and
30 in the elderly. Due to the incomplete resistance to RSV reinfection induced by natural infection, RSV may infect multiple times during childhood and life. The goal of RSV immunoprophylaxis is to induce sufficient resistance to prevent the serious disease which may be associated with RSV
35 infection. The current strategies for developing RSV vaccines principally revolve around the administration of purified viral antigen or the development of live attenuated RSV for

subgroups of RSV A and B is mainly linked to the G glycoprotein, whereas the F glycoprotein is more closely related between the subgroups.

Despite decades of research, no safe and effective RSV vaccine has been developed for the prevention of severe morbidity and mortality associated with RSV infection. A formalin-inactivated virus vaccine has failed to provide protection against RSV infection and its exacerbated symptoms during subsequent infection by the wild-type virus in infants (Kapikian et al., 1969, Am. J. Epidemiol. 89:405-21; Chin et al., 1969, Am. J. Epidemiol. 89:449-63). Efforts since have focused on developing live attenuated temperature-sensitive mutants by chemical mutagenesis or cold passage of the wild-type RSV (Gharpure et al., 1969, J. Virol. 3: 414-21; Crowe et al., 1994, Vaccine 12: 691-9). However, earlier trials yielded discouraging results with these live attenuated temperature sensitive mutants. Virus candidates were either underattenuated or overattenuated (Kim et al., 1973, Pediatrics 52:56-63; Wright et al., 1976, J. Pediatrics 88:931-6) and some of the vaccine candidates were genetically unstable which resulted in the loss of the attenuated phenotype (Hodes et al., 1974, Proc. Soc. Exp. Biol. Med. 145:1158-64).

Attempts have also been made to engineer recombinant vaccinia vectors which express RSV F or G envelope glycoproteins. However, the use of these vectors as vaccines to protect against RSV infection in animal studies has shown inconsistent results (Olmsted et al. 1986, Proc. Natl. Acad. Sci. 83:7462-7466; Collins et al., 1990, Vaccine 8:164-168).

Thus, efforts have turned to engineering recombinant RSV to generate vaccines. For a long time, negative-sense RNA viruses were refractory to study. Only recently has it been possible to recover negative strand RNA viruses using a recombinant reverse genetics approach (U.S. Patent No. 5,166,057 to Palese et al.). Although this method was originally applied to engineer influenza viral genomes (Luytjes et al. 1989, Cell 59:1107-1113; Enami et al. 1990,

Proc. Natl. Acad. Sci. USA 92: 11563-11567), it has been successfully applied to a wide variety of segmented and nonsegmented negative strand RNA viruses, including rabies (Schnell et al. 1994, EMBO J. 13: 4195-4203); VSV (Lawson et al., 1995, Proc. Natl. Acad. Sci. USA 92: 4477-81); measles virus (Radecke et al., 1995, EMBO J. 14:5773-84); rinderpest virus (Baron & Barrett, 1997, J. virol. 71: 1265-71); human parainfluenza virus (Hoffman & Banerjee, 1997, J. Virol. 71:3272-7; Dubin et al., 1997, Virology 235:323-32); SV5 (He et al., 1997, Virology 237:249-60); respiratory syncytial virus (Collins et al. 1991, Proc. Natl. Acad. Sci. USA 88: 9663-9667) and Sendai virus (Park et al. 1991, Proc. Natl. Acad. Sci. USA 88:5537-5541; Kato et al. 1996, Genes to Cells 1:569-579). Although this approach has been used to successfully rescue RSV, a number of groups have reported that RSV is still refractory to study given several properties of RSV which distinguish it from the better characterized paramyxoviruses of the genera Paramyxovirus, Rubulavirus, and Morbillivirus. These differences include a greater number of RNAs, an unusual gene order at the 3' end of the genome, extensive strain-to-strain sequence diversity, several proteins not found in other nonsegmented negative strand RNA viruses and a requirement for the M2 protein (ORF1) to proceed with full processing of full length transcripts and rescue of a full length genome (Collins et al. PCT WO97/12032; Collins, P.L. et al. pp 1313-1357 of volume 1, Fields Virology, et al., Eds. (3rd ed., Raven Press, 1996).

3. SUMMARY OF THE INVENTION

The present invention relates to genetically engineered recombinant RS viruses and viral vectors which contain heterologous genes which for the use as vaccines. In accordance with the present invention, the recombinant RS viral vectors and viruses are engineered to contain heterologous genes, including genes of other viruses, pathogens, cellular genes, tumor antigens, or to encode combinations of genes from different strains of RSV.

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Recombinant negative-strand viral RNA templates are described which may be used to transfect transformed cell that express the RNA dependent RNA polymerase and allow for complementation. Alternatively, a plasmid expressing the components of the RNA polymerase from an appropriate promoter can be used to transfect cells to allow for complementation of the negative-strand viral RNA templates. Complementation may also be achieved with the use of a helper virus or wild-type virus to provide the RNA dependent RNA polymerase. The RNA templates are prepared by transcription of appropriate DNA sequences with a DNA-directed RNA polymerase. The resulting RNA templates are of negative-or positive-polarity and contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template. Bicistronic mRNAs can be constructed to permit internal initiation of translation of viral sequences and allow for the expression of foreign protein coding sequences from the regular terminal initiation site, or vice versa.

As demonstrated by the examples described herein, recombinant RSV genome in the positive-sense or negative-sense orientation is co-transfected with expression vectors encoding the viral nucleocapsid (N) protein, the associated nucleocapsid phosphoprotein (P), the large (L) polymerase subunit protein, with or without the M2/ORF1 protein of RSV to generate infectious viral particles. Vaccinia vectors expressing RSV virus polypeptides are used as the source of proteins which were able to replicate and transcribe synthetically derived RNPs. The minimum subset of RSV proteins needed for specific replication and expression of the viral RNP was found to be the three polymerase complex proteins (N, P and L). This suggests that the M2 gene function is not absolutely required for the replication, expression and rescue of infectious RSV.

The expression products and/or chimeric virions obtained may advantageously be utilized in vaccine formulations. In particular, recombinant RSV genetically engineered to demonstrate an attenuated phenotype may be utilized as a live

vRNA = genomic virus RNA

viral polymerase complex = PA, PB1, PB2 and NP

WSN = influenza A/WSN/33 virus

WSN-HK virus: reassortment virus containing seven genes
5 from WSN virus and the NA gene from
influenza A/HK/8/68 virus

4. DESCRIPTION OF THE FIGURES

FIG. 1. Schematic representation of the RSV/CAT
10 construct (pRSVA2CAT) used in rescue experiments. The
approximate 100 nt long leader and 200 nt long trailer regions
of RSV were constructed by the controlled annealing of
synthetic oligonucleotides containing partial overlapping
complementarity. The overlapping leader oligonucleotides are
15 indicated by the 1L - 5L shown in the construct. The
overlapping trailer nucleotides are indicated by the 1T - 9T
shown in the construct. The nucleotide sequences of the
leader and trailer DNAs were ligated into purified CAT gene
DNA at the indicate XbaI and PstI sites respectively. This
20 entire construct was then ligated into KpnI/HindIII digested
pUC19. The inclusion of a T7 promoter sequence and a HgaI
site flanking the trailer and leader sequences, respectively,
allowed in vitro synthesis of RSV/CAT RNA transcripts
containing the precise genomic sequence 3' and 5' ends.

25

FIG. 2. Thin layer chromatogram (TLC) showing the CAT
activity present in 293 cell extracts following infection and
transfection with RNA transcribed from the RSV/CAT construct
shown in Figure 11. Confluent monolayers of 293 cells in six-
30 well plates (-10^6 cells) were infected with either RSV A2 or
B9320 at an m.o.i. of 0.1-1.0 pfu cell. At 1 hour post
infection cells were transfected with 5-10 μ g of CAT/RSV using
the Transfect-ActTM protocol of Life Technologies. At 24 hours
post infection the infected/transfected monolayers were
35 harvested and processed for subsequence CAT assay according to
Current Protocols in Molecular Biology, Vol. 1, Chapter 9.6.2;
Gorman, et al., (1982) Mol. Cell. Biol. 2:1044-1051. Lanes 1,

2, 3 and 4 show the CAT activity present in (1) uninfected 293 cells, transfected with CAT/RSV-A2 infected 293 cells, co-infected with supernatant from (2) above. The CAT activity observed in each lane was produced from 1/5 of the total cellular extract from 10^6 cells.

FIG. 3. Schematic representation of the RSV strain A2 genome showing the relative positions of the primer pairs used for the synthesis of cDNAs comprising the entire genome. The endonuclease sites used to splice these clones together are indicated; these sites were present in the native RSV sequence and were included in the primers used for cDNA synthesis. Approximately 100 ng of viral genomic RNA was used in RT/PCR reactions for the separate synthesis of each of the seven cDNAs. The primers for the first and second strand cDNA synthesis from the genomic RNA template are also shown. For each cDNA, the primers for the first strand synthesis are nos. 1-7 and the primers for the second strand synthesis are nos. 1'-7'.

20

FIG. 4. Schematic representation of the RSV subgroup B strain B9320. BamHI sites were created in the oligonucleotide primers used for RT/PCR in order to clone the G and F genes from the B9320 strain into RSV subgroup A2 antigenomic cDNA (FIG. 4A). A cDNA fragment which contained G and F genes from 4326 nucleotides to 9387 nucleotides of A2 strain was first subcloned into pUC19 (pUCRVH). Bgl II sites were created at positions of 4630 (SH/G intergenic junction FIG. 4B) and 7554 (F/M2 intergenic junction (FIG. 4C). B93260 A-G and -F cDNA inserted into pUCR/H which is deleted of the A-G and F genes. The resulting antigenomic cDNA clone was termed as pRSVB-GF and was used to transfect Hep-2 cells to generate infectious RSVB-GF virus.

FIG. 5. Recombinant RSVB-GF virus was characterized by RT/PCR using RSV subgroup B specific primers. RSV subgroup B specific primers in the G region were incubated with aliquots

of the recombinant RSV viral genomes and subjected to PCR. The PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. As shown, no DNA product was produced in the RT/PCR reaction using RSV A2 as a template. However, a predicted product of 254 base pairs was seen in RT/PCR of RSVB-GF RNA and PCR control of plasmid pRSV-GF DNA as template, indicating the rescued virus contained G and F genes derived from B9320 virus.

10

FIG. 6. Identification of chimeric (rRSVA2(B-G) by RT/PCR and Northern blot analysis of RNA expression. FIG. 6A. RT/PCT analysis of chimeric rRSV A2(B-G), A2(B-G), in comparison with wild-type A2(A2). Virion RNA extracted from rRSVA2(B-G) (lanes 1, 2) and rRSVA2 (lanes 3,4) was reverse transcribed using a primer annealed to (-) sense vRNA in the RSV F gene in the presence (+) or absence (-) of reverse transcriptase (RT), followed by PCR with a primer pair flanking the B-G insertion site. No DNA was detected in RT/PCR when reverse transcriptase (RT) was absent (lanes 2,4). A cDNA fragment, which is about 1kb bigger than the cDNA derived from A2, was produced from rRSVA(B-G). This longer PCR DNA product was digested by *Stu I* restriction enzyme unique to the inserted B-G gene (lane 5). 100 bp DNA size marker is indicated (M). FIG. 6B. Northern blot analysis of G mRNA expression. Hep-2 cells were infected with RSV B9320, rRSV and chimeric rRSV A2 (B-G). At 48 hr postinfection, total cellular RNA was extracted and electrophoresed on a 1.2% agarose gel containing formaldehyde. RNA was transferred to Hybond Nylon membrane and the filter was hybridized with a ³²P-labeled oligonucleotide probe specific for A2-G or specific for B9320-G mRNA. Both A2 G specific and B9320 G specific transcripts were detected in the rRSV A2 (B-G) infected cells. The run-off RNA transcript (G-M2) from rRSV A2 (B-G) infected cells is also indicated.

FIG. 7. Analysis of protein expression by rRSV A2 (B-G). Hep-2 cells were mock-infected (lanes 1, 5), infected with RSV B9320 (lanes 2, 6), rRSV (lanes 3, 7) and rRSV A2 (B-G) (lanes 4, 8). At 14-18 hr postinfection, infected cells were labeled with ^{35}S -promix and polypeptides were immunoprecipitated by goat polyclonal antiserum against RSV A2 strain (lanes 1-5) or by mouse polyclonal antiserum against RSV B9320 strain (lanes 5-8). Immunoprecipitated polypeptides were separated on a 10% polyacrylamide gel. Both RSV A2 specific G protein and RSV B9320 specific G protein were produced in rRSV A2 (B-G) infected cells. The G protein migration is indicated by *. Mobility of the F1 glycoprotein, and N, P, and M is indicated. Molecular sizes are shown on the left in kilodaltons.

FIG. 8. Plaque morphology of rRSV, rRSVC3G, rRSV A2 (B-G) and wild-type A2 virus (wt A2). Hep-2 cells were infected with each virus and incubated at 35°C for six days. The cell monolayers were fixed, visualized by immunostaining, and photographed.

FIG. 9. Growth curve of rRSV, rRSVC4G, wild-type A2 RSV (wt A2) and chimeric rRSV A2 (B-G). Hep-2 cells were infected with either virus at a moi of 0.5 and the medium was harvested at 24 hr intervals. The titer of each virus was determined in duplicate by plaque assay on Hep-2 cells and visualized by immunostaining.

FIG. 10. RSV L protein charged residue clusters targeted for site-directed mutagenesis. Charged amino acid residues in contiguous clusters were converted to alanines by site-directed mutagenesis of the RSV L gene using the QuikChange site-directed mutagenesis kit (Stratagene).

FIG. 11. RSV L protein cysteine residues targeted for site-directed mutagenesis. Cysteine residues were converted to alanine-residues by site-directed mutagenesis of the RSV L

gene using the QuikChange site-directed mutagenesis kit (Stratagene).

FIG. 12. Identification RSV M2-2 and SH deletion mutants. Deletions in M2-2 were generated by Hind III digestion of pET(S/B) followed by recloning of a remaining Sac I to BamHI fragment into a full-length clone. Deletions in SH were generated by Sac I digestion of pET(A/S) followed by recloning of a remaining Avr II Sac I fragment into a full-length clone. FIG. 12A. Identification of the recovered rRSVsSH and rRSV M2-2 was performed by RT/PCR using primer pairs specific for the SH gene or M2-2 gene, respectively. Fig. 12B rRSV SH M2-2 was also detected by RT/PCR using primer pairs specific for the M2-2 and SH genes. RT/PCR products were run on an ethidium bromide agarose gel and bands were visualized by ultraviolet (UV) light.

5. DESCRIPTION OF THE INVENTION

The present invention relates to genetically engineered recombinant RS viruses and viral vectors which express heterologous genes or mutated RS viral genes or a combination of viral genes derived from different strains of RS virus. The invention relates to the construction and use of recombinant negative strand RS viral RNA templates which may be used with viral RNA-directed RNA polymerase to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. The RNA templates of the present invention may be prepared by transcription of appropriate DNA sequences using a DNA-directed RNA polymerase such as bacteriophage T7, T3 or Sp6 polymerase. The recombinant RNA templates may be used to transfect continuous/transfected cell lines that express the RNA-directed RNA polymerase proteins allowing for complementation.

The invention is demonstrated by way of working examples in which infectious RSV is rescued from cDNA containing the

RSV genome in the genomic or antigenomic sense introduced into cells expressing the N, P, and L proteins of the RSV polymerase complex. The working examples further demonstrate that expression of M2-1 is not required for recovery of
5 infectious RSV from cDNA which is contrary to what has been reported earlier (Collins et al., 1995, Proc. Natl. Acad. Sci. USA 92:11563-7). Furthermore, the addition of plasmids expressing M2-1 has little effect on the RSV rescue efficiency. M2-deleted-RSV is an excellent vehicle to
10 generate chimeric RSV encoding heterologous gene products in place of the M2 genes, these chimeric viral vectors and rescued virus particles have utility as expression vectors for the expression of heterologous gene products and as live attenuated RSV vaccines expressing either RSV antigenic
15 polypeptides or antigenic polypeptides of other viruses.

The invention is further demonstrated by way of working examples in which a cDNA clone which contained the complete genome of RSV, in addition to a T7 promoter, a hepatitis delta virus ribozyme and a T7 terminator is used to generate an
20 infectious viral particle when co-transfected with expression vectors encoding the N, P, L and M2/orf1 proteins of RSV. In addition, the working examples describe RNA transcripts of cloned DNA containing the coding region -- in negative sense orientation -- of the chloramphenicol-acetyl-transferase (CAT)
25 gene or the green fluorescent protein (GFP) gene flanked by the 5' terminal and 3' terminal nucleotides of the RSV genome. The working examples further demonstrate that an RSV promoter mutated to have increased activity resulted in rescue of infectious RSV particles from a full length RSV cDNA with high
30 efficiency. These results demonstrate the successful use of recombinant viral negative strand templates and RSV polymerase with increased activity to rescue RSV. This system is an excellent tool to engineer RSV viruses with defined biological properties, e.g. live-attenuated vaccines against RSV, and to
35 use recombinant RSV as an expression vector for the expression of heterologous gene products.

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This invention relates to the construction and use of recombinant negative strand viral RNA templates which may be used with viral RNA-directed RNA polymerase to express heterologous gene products in appropriate host cells, to
5 rescue the heterologous gene in virus particles and/or express mutated or chimeric recombinant negative strand viral RNA templates (see U.S. Patent No. 5,166,057 to Palese et al., incorporated herein by reference in its entirety). In a specific embodiment of the invention, the heterologous gene
10 product is a peptide or protein derived from another strain of the virus or another virus. The RNA templates may be in the positive or negative-sense orientation and are prepared by transcription of appropriate DNA sequences using a DNA-directed RNA polymerase such as bacteriophage T7, T3 or the
15 Sp6 polymerase.

The ability to reconstitute RNP's in vitro allows the design of novel chimeric influenza and RSV viruses which express foreign genes. One way to achieve this goal involves modifying existing viral genes. For example, the HA gene of
20 influenza may be modified to contain foreign sequences in its external domains. Where the heterologous sequence are epitopes or antigens of pathogens, these chimeric viruses may be used to induce a protective immune response against the disease agent from which these determinants are derived.

25 For example, a chimeric RNA may be constructed in which a coding sequence derived from the gp120 coding region of human immunodeficiency virus was inserted into the F or G coding sequence of influenza, and chimeric virus was produced from transfection of this chimeric RNA segment into a host cell
30 infected with wild-type RSV.

In addition to modifying genes coding for surface proteins, genes coding for nonsurface proteins may be altered. The latter genes have been shown to be associated with most of the important cellular immune responses in the RS virus
35 system. Thus, the inclusion of a foreign determinant in the G or F gene of RSV may - following infection - induce an effective cellular immune response against this determinant.

Such an approach may be particularly helpful in situations in which protective immunity heavily depends on the induction of cellular immune responses (e.g., malaria, etc.).

The present invention also relates to attenuated

- 5 recombinant RSV produced by introducing specific mutations in the genome of RSV which results in an amino acid change in an RSV protein, such as a polymerase protein, which results in an attenuated phenotype.

10 **5.1. CONSTRUCTION OF THE RECOMBINANT RNA TEMPLATES**

Heterologous gene coding sequences flanked by the complement of the viral polymerase binding site/promoter, e.g., the complement of the 3'-RSV termini or the 3'- and 5'- RSV termini may be constructed using techniques known in the art.

- 15 Heterologous gene coding sequences may also be flanked by the complement of the RSV polymerase binding site/promoter, e.g., the leader and trailer sequence of RSV using techniques known in the art. Recombinant DNA molecules containing these hybrid sequences can be cloned and transcribed by a DNA-directed RNA
20 polymerase, such as bacteriophage T7, T3 or the Sp6 polymerase and the like, to produce the recombinant RNA templates which possess the appropriate viral sequences that allow for viral polymerase recognition and activity.

- In a preferred embodiment of the present invention, the
25 heterologous sequences are derived from the genome of another strain of RSV, e.g., the genome of RSV A strain is engineered to include the nucleotide sequences encoding the antigenic polypeptides G and F of RSV B strain, or fragments thereof. In such an embodiment of the invention, heterologous coding
30 sequences from another strain of RSV can be used to substitute for nucleotide sequences encoding antigenic polypeptides of the starting strain, or be expressed in addition to the antigenic polypeptides of the parent strain, so that a recombinant RSV genome is engineered to express the antigenic
35 polypeptides of one, two or more strains of RSV.

In yet another embodiment of the invention, the heterologous sequences are derived from the genome of any

strain of influenza virus. In accordance with the present invention, the heterologous coding sequences of influenza may be inserted within a RSV coding sequence such that a chimeric gene product is expressed which contains the heterologous peptide sequence within the RSV viral protein. In either embodiment, the heterologous sequences derived from the genome of influenza may include, but are not limited to HA, NA, PB1, PB2, PA, NS1 or NS2.

In one specific embodiment of the invention, the heterologous sequences are derived from the genome of human immunodeficiency virus (HIV), preferably human immunodeficiency virus-1 or human immunodeficiency virus-2. In another embodiment of the invention, the heterologous coding sequences may be inserted within an influenza gene coding sequence such that a chimeric gene product is expressed which contains the heterologous peptide sequence within the influenza viral protein. In such an embodiment of the invention, the heterologous sequences may also be derived from the genome of a human immunodeficiency virus, preferably of human immunodeficiency virus-1 or human immunodeficiency virus-2.

In instances whereby the heterologous sequences are HIV-derived, such sequences may include, but are not limited to sequences derived from the env gene (i.e., sequences encoding all or part of gp160, gp120, and/or gp41), the pol gene (i.e., sequences encoding all or part of reverse transcriptase, endonuclease, protease, and/or integrase), the gag gene (i.e., sequences encoding all or part of p7, p6, p55, p17/18, p24/25) tat, rev, nef, vif, vpu, vpr, and/or vpx.

One approach for constructing these hybrid molecules is to insert the heterologous coding sequence into a DNA complement of a RSV genomic RNA so that the heterologous sequence is flanked by the viral sequences required for viral polymerase activity; i.e., the viral polymerase binding site/promoter, hereinafter referred to as the viral polymerase binding site. In an alternative approach, oligonucleotides encoding the viral polymerase binding site, e.g., the

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complement of the 3'-terminus or both termini of the virus genomic segments can be ligated to the heterologous coding sequence to construct the hybrid molecule. The placement of a foreign gene or segment of a foreign gene within a target
5 sequence was formerly dictated by the presence of appropriate restriction enzyme sites within the target sequence. However, recent advances in molecular biology have lessened this problem greatly. Restriction enzyme sites can readily be placed anywhere within a target sequence through the use of
10 site-directed mutagenesis (e.g., see, for example, the techniques described by Kunkel, 1985, Proc. Natl. Acad. Sci. U.S.A. 82;488). Variations in polymerase chain reaction (PCR) technology, described infra, also allow for the specific insertion of sequences (i.e., restriction enzyme sites) and
15 allow for the facile construction of hybrid molecules. Alternatively, PCR reactions could be used to prepare recombinant templates without the need of cloning. For example, PCR reactions could be used to prepare double-stranded DNA molecules containing a DNA-directed RNA
20 polymerase promoter (e.g., bacteriophage T3, T7 or Sp6) and the hybrid sequence containing the heterologous gene and the influenza viral polymerase binding site. RNA templates could then be transcribed directly from this recombinant DNA. In yet another embodiment, the recombinant RNA templates may be
25 prepared by ligating RNAs specifying the negative polarity of the heterologous gene and the viral polymerase binding site using an RNA ligase. Sequence requirements for viral polymerase activity and constructs which may be used in accordance with the invention are described in the subsections
30 below.

5.1.1. INSERTION OF THE HETEROLOGOUS GENES

The genes coding for the M2 or L proteins contain a single open reading frame. The gene coding for NS contains
35 two open reading frames for NS1 and NS2. The G and F proteins, coded for by separate genes, are the major surface glycoproteins of the virus. Consequently, these proteins are

the major targets for the humoral immune response after infection. Insertion of a foreign gene sequence into any of these coding regions could be accomplished by either a complete replacement of the viral coding region with the
5 foreign gene or by a partial replacement. Complete replacement would probably best be accomplished through the use of PCR-directed mutagenesis.

Alternatively, a bicistronic mRNA could be constructed to permit internal initiation of translation of viral sequences
10 and allow for the expression of foreign protein coding sequences from the regular terminal initiation site. Alternatively, a bicistronic mRNA sequence may be constructed wherein the viral sequence is translated from the regular terminal open reading frame, while the foreign sequence is
15 initiated from an internal site. Certain internal ribosome entry site (IRES) sequences may be utilized. The IRES sequences which are chosen should be short enough to not interfere with RS virus packaging limitations. Thus, it is preferable that the IRES chosen for such a bicistronic
20 approach be no more than 500 nucleotides in length, with less than 250 nucleotides being preferred. Further, it is preferable that the IRES utilized not share sequence or structural homology with picornaviral elements. Preferred IRES elements include, but are not limited to the mammalian
25 BiP IRES and the hepatitis C virus IRES.

5.2. EXPRESSION OF HETEROLOGOUS GENE PRODUCTS USING RECOMBINANT RNA TEMPLATE

The recombinant templates prepared as described above can
30 be used in a variety of ways to express the heterologous gene products in appropriate host cells or to create chimeric viruses that express the heterologous gene products. In one embodiment, the recombinant template can be combined with viral polymerase complex purified *infra*, to produce rRNPs
35 which are infectious. To this end, the recombinant template can be transcribed in the presence of the viral polymerase complex. Alternatively, the recombinant template may be mixed

with or transcribed in the presence of viral polymerase complex prepared using recombinant DNA methods (e.g. see Kingsbury et al., 1987, Virology 156:396-403). In yet another embodiment, the recombinant template can be used to transfect appropriate host cells to direct the expression of the heterologous gene product at high levels. Host cell systems which provide for high levels of expression include continuous cell lines that supply viral functions such as cell lines superinfected with RSV, cell lines engineered to complement RSV viral functions, etc.

5.3. PREPARATION OF CHIMERIC NEGATIVE STRAND RNA VIRUS

In order to prepare chimeric virus, reconstituted RNPs containing modified RSV RNAs or RNA coding for foreign proteins may be used to transfect cells which are also infected with a "parent" RSV virus. Alternatively, the reconstituted RNP preparations may be mixed with the RNPs of wild type parent virus and used for transfection directly. Following reassortment, the novel viruses may be isolated and their genomes be identified through hybridization analysis. In additional approaches described herein for the production of infectious chimeric virus, rRNPs may be replicated in host cell systems that express the RSV or influenza viral polymerase proteins (e.g., in virus/host cell expression systems; transformed cell lines engineered to express the polymerase proteins, etc.), so that infectious chimeric virus are rescued; in this instance, helper virus need not be utilized since this function is provided by the viral polymerase proteins expressed. In a particularly desirable approach, cells infected with rRNPs engineered for all eight influenza virus segments may result in the production of infectious chimeric virus which contain the desired genotype; thus eliminating the need for a selection system.

Theoretically, one can replace any one of the genes of RSV, or part of any one of the RSV genes, with the foreign sequence. However, a necessary part of this equation is the

ability to propagate the defective virus (defective because a normal viral gene product is missing or altered). A number of possible approaches exist to circumvent this problem.

A third approach to propagating the recombinant virus may involve co-cultivation with wild-type virus. This could be done by simply taking recombinant virus and co-infecting cells with this and another wild-type virus (preferably a vaccine strain). The wild-type virus should complement for the defective virus gene product and allow growth of both the wild-type and recombinant virus. This would be an analogous situation to the propagation of defective-interfering particles of influenza virus (Nayak et al., 1983, In: Genetics of Influenza Viruses, P. Palese and D. W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 255-279). In the case of defective-interfering viruses, conditions can be modified such that the majority of the propagated virus is the defective particle rather than the wild-type virus. Therefore this approach may be useful in generating high titer stocks of recombinant virus. However, these stocks would necessarily contain some wild-type virus.

Alternatively, synthetic RNPs may be replicated in cells co-infected with recombinant viruses that express the RS virus polymerase proteins. In fact, this method may be used to rescue recombinant infectious virus in accordance with the invention. To this end, the RSV virus polymerase proteins may be expressed in any expression vector/host cell system, including but not limited to viral expression vectors (e.g., vaccinia virus, adenovirus, baculovirus, etc.) or cell lines that express the polymerase proteins (e.g., see Krystal et al., 1986, Proc. Natl. Acad. Sci. USA 83: 2709-2713).

5.4. GENERATION OF CHIMERIC VIRUSES WITH AN ATTENUATED PHENOTYPE

The methods of present invention may be used to introduce mutations or heterologous sequences to generate chimeric attenuated viruses which have many applications, including analysis of RSV molecular biology, pathogenesis, and growth

and infection properties. In accordance with the present invention, mutations or heterologous sequences may be introduced for example into the F or G protein coding sequences, NS1, NS2, M1ORF, M2ORF, N, P, or L coding sequences. In yet another embodiment of the present invention, a particular viral gene, or the expression thereof, may be eliminated to generate an attenuated phenotype, e.g., the M ORF may be deleted from the RSV genome to generate a recombinant RSV with an attenuated phenotype. In yet another embodiment, the individual internal genes of human RSV can be replaced by another strains counterpart, or their bovine or murine counterpart. This may include part or all of one or more of the NS1, NS2, N, P, M, SH, M2(ORF1), M2(ORF2) and L genes or the G and F genes. The RSV genome contains ten mRNAs encoding three transmembrane proteins, G protein, fusion F protein required for penetration, and the small SH protein; the nucleocapsid proteins N, P and L; transcription elongation factor M2 ORF 1; the matrix M protein and two nonstructural proteins, NS1 and NS2. Any one of the proteins may be targeted to generate and attenuated phenotype. Other mutations which may be utilized to result in an attenuated phenotype are insertional, deletional and site directed mutations of the leader and trailer sequences.

In accordance with the present invention, an attenuated RSV exhibits a substantially lower degree of virulence as compared to a wild-type virus, including a slower growth rate, such that the symptoms of viral infection do not occur in an immunized individual.

In accordance with the present invention attenuated recombinant RSV may be generated by incorporating a broad range of mutations including single nucleotide changes, site-specific mutations, insertions, substitutions, deletions, or rearrangements. These mutations may affect a small segment of the RSV genome, e.g., 15 to 30 nucleotides, or large segments of the RSV genome, e.g., 50 to 1000 nucleotides, depending on the nature of the mutation. In yet another embodiment, mutations are introduced upstream or downstream of an existing

generated during replication. Since the number of infectious particles produced during replication are reduced, the altered viruses demonstrated attenuated characteristics. However, the number of antigenic virus particles produced will be

5 sufficient to induce a vigorous immune response.

An alternative way to engineer attenuated viruses involves the introduction of an alteration, including but not limited to an insertion, deletion or substitution of one or more amino acid residues and/or epitopes into one or more of
10 the viral proteins. This may be readily accomplished by engineering the appropriate alteration into the corresponding viral gene sequence. Any change that alters the activity of the viral protein so that viral replication is modified or reduced may be accomplished in accordance with the invention.

15 For example, alterations that interfere with but do not completely abolish viral attachment to host cell receptors and ensuing infection can be engineered into viral surface antigens or viral proteases involved in processing to produce an attenuated strain. According to this embodiment, viral
20 surface antigens can be modified to contain insertions, substitution or deletions of one or more amino acids or epitopes that interfere with or reduce the binding affinity of the viral antigen for the host cell receptors. This approach offers an added advantage in that a chimeric virus which
25 expresses a foreign epitope may be produced which also demonstrates attenuated characteristics. Such viruses are ideal candidates for use as live recombinant vaccines. For example, heterologous gene sequences that can be engineered into the chimeric viruses of the invention include, but are
30 not limited to, epitopes of human immunodeficiency virus (HIV) such as gp120; hepatitis B virus surface antigen (HBsAg); the glycoproteins of herpes virus (e.g., gD, gE); VP1 of poliovirus; and antigenic determinants of nonviral pathogens such as bacteria and parasites, to name but a few.

35 In this regard, RSV is an ideal system in which to engineer foreign epitopes, because the ability to select from thousands of virus variants for constructing chimeric viruses

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obviates the problem of host resistance or immune tolerance encountered when using other virus vectors such as vaccinia.

In another embodiment, alterations of viral proteases required for processing viral proteins can be engineered to produce attenuation. Alterations which affect enzyme activity and render the enzyme less efficient in processing, should affect viral infectivity, packaging, and/or release to produce an attenuated virus.

In another embodiment, viral enzymes involved in viral replication and transcription of viral genes, e.g., viral polymerases, replicases, helicases, etc. may be altered so that the enzyme is less efficient or active. Reduction in such enzyme activity may result in the production of fewer progeny genomes and/or viral transcripts so that fewer infectious particles are produced during replication.

The alterations engineered into any of the viral enzymes include but are not limited to insertions, deletions and substitutions in the amino acid sequence of the active site of the molecule. For example, the binding site of the enzyme could be altered so that its binding affinity for substrate is reduced, and as a result, the enzyme is less specific and/or efficient. For example, a target of choice is the viral polymerase complex since temperature sensitive mutations exist in all polymerase proteins. Thus, changes introduced into the amino acid positions associated with such temperature sensitivity can be engineered into the viral polymerase gene so that an attenuated strain is produced.

5.4.1. THE RSV L GENE AS A TARGET FOR ATTENUATION

In accordance with the present invention, the RSV L gene is an important target to generate recombinant RSV with an attenuated phenotype. The L gene represents 48% of the entire RSV genome. The present invention encompasses generating L gene mutants with defined mutations or random mutations in the RSV L gene. Any number of techniques known to those skilled in the art may be used to generate both defined or random

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In yet another approach to generate mutants with an attenuated phenotype utilizes a scanning mutagenesis approach to mutate cysteines to amino acids, such as glycines or alanines. Such an approach takes advantage of the frequent
5 role of cysteines in intramolecular and intermolecular bond formations, thus by mutating cysteines to another residue, such as a conservative substitution e.g., valine or alanine, or a drastic substitution e.g., aspartic acid, the stability and function of a protein may be altered due to disruption of
10 the protein's tertiary structure. There are approximately thirty-nine cysteine residues present in the RSV L gene.

In yet another approach random mutagenesis of the RSV L gene will cover residues other than charged or cysteines. Since the RSV L gene is very large, such an approach may be
15 accomplished by mutagenizing large cDNA fragments of the L gene by PCR mutagenesis. The functionality of such mutants may be screened by a minigenome replication system and the recovered mutants are then further analyzed in vitro and in vivo.

20

5.5. VACCINE FORMULATIONS USING THE CHIMERIC VIRUSES

Virtually any heterologous gene sequence may be constructed into the chimeric viruses of the invention for use
25 in vaccines. In a preferred embodiment, the present invention relates to bivalent RSV vaccines which confers protection against RSV-A and RSV-B. To formulate such a vaccine, a chimeric RS virus is used which expresses the antigenic polypeptides of both RSV-A and RSV-B subtypes. In yet another
30 preferred embodiment, the present invention relates to a bivalent vaccine which confers protection against both RSV and influenza. To formulate such a vaccine, a chimeric RS virus is used which expresses the antigenic polypeptides of both RSV and influenza.

35 Preferably, epitopes that induce a protective immune response to any of a variety of pathogens, or antigens that bind neutralizing antibodies may be expressed by or as part of

the chimeric viruses. For example, heterologous gene sequences that can be constructed into the chimeric viruses of the invention for use in vaccines include but are not limited to sequences derived from a human immunodeficiency virus

5 (HIV), preferably type 1 or type 2. In a preferred embodiment, an immunogenic HIV-derived peptide which may be the source of an antigen may be constructed into a chimeric influenza virus that may then be used to elicit a vertebrate immune response.

10 Such HIV-derived peptides may include, but are not limited to sequences derived from the env gene (i.e., sequences encoding all or part of gp160, gp120, and/or gp41), the pol gene (i.e., sequences encoding all or part of reverse transcriptase, endonuclease, protease, and/or integrase), the
15 gag gene (i.e., sequences encoding all or part of p7, p6, p55, p17/18, p24/25), tat, rev, nef, vif, vpu, vpr, and/or vpx.

Other heterologous sequences may be derived from hepatitis B virus surface antigen (HBsAg); the glycoproteins of herpes virus (e.g. gD, gE); VP1 of poliovirus; antigenic
20 determinants of non-viral pathogens such as bacteria and parasites, to name but a few. In another embodiment, all or portions of immunoglobulin genes may be expressed. For example, variable regions of anti-idiotypic immunoglobulins that mimic such epitopes may be constructed into the chimeric
25 viruses of the invention.

Either a live recombinant viral vaccine or an inactivated recombinant viral vaccine can be formulated. A live vaccine may be preferred because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that
30 occurring in natural infections, and therefore, confers substantial, long-lasting immunity. Production of such live recombinant virus vaccine formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in the allantois of the chick embryo
35 followed by purification.

In this regard, the use of genetically engineered RSV (vectors) for vaccine purposes may require the presence of

formalin or other agents used in the manufacturing of killed virus vaccines.

In another embodiment of this aspect of the invention, inactivated vaccine formulations may be prepared using
5 conventional techniques to "kill" the chimeric viruses. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity of the virus is destroyed without affecting its immunogenicity. In order to prepare inactivated vaccines, the chimeric virus
10 may be grown in cell culture or in the allantois of the chick embryo, purified by zonal ultracentrifugation, inactivated by formaldehyde or β -propiolactone, and pooled. The resulting vaccine is usually inoculated intramuscularly.

Inactivated viruses may be formulated with a suitable
15 adjuvant in order to enhance the immunological response. Such adjuvants may include but are not limited to mineral gels, e.g., aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols, polyanions; peptides; oil emulsions; and potentially useful human adjuvants such as BCG
20 and *Corynebacterium parvum*.

Many methods may be used to introduce the vaccine formulations described above, these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. It may be
25 preferable to introduce the chimeric virus vaccine formulation via the natural route of infection of the pathogen for which the vaccine is designed. Where a live chimeric virus vaccine preparation is used, it may be preferable to introduce the formulation via the natural route of infection for influenza
30 virus. The ability of RSV and influenza virus to induce a vigorous secretory and cellular immune response can be used advantageously. For example, infection of the respiratory tract by chimeric RSV or influenza viruses may induce a strong secretory immune response, for example in the urogenital
35 system, with concomitant protection against a particular disease causing agent.

The following sections describe by way of example, and

not by limitation, the manipulation of the negative strand RNA viral genomes using RSV as an example to demonstrate the applicability of the methods of the present invention to generate chimeric viruses for the purposes of heterologous gene expression, generating infectious viral particles and attenuated viral particles for the purposes of vaccination.

6. RESCUE OF INFECTIOUS RESPIRATORY SYNCYTIAL VIRUSES (RSV) USING RNA DERIVED FROM SPECIFIC RECOMBINANT DNAs

10 This example describes a process for the rescue of infectious respiratory syncytial virus (RSV), derived from recombinant cDNAs encoding the entire RSV RNA genome into stable and infectious RSVs, as noted in Section 5 above. The method described may be applied to both segmented and non-
15 segmented RNA viruses, including orthomyxovirus, paramyxovirus, e.g., Sendai virus, parainfluenza virus types 1-4, mumps, newcastle disease virus; morbillivirus, e.g., measles, canine distemper virus, rinderpest virus; pneumovirus, e.g., respiratory syncytial virus; rhabdovirus,
20 e.g., rabies, vesiculovirus, vesicular stomatitis virus; but is described by way of example in terms of RSV. This process can be used in the production of chimeric RSV viruses which can express foreign genes, i.e., genes non-native to RSV, including other viral proteins such as the HIV env protein.
25 Another exemplary way to achieve the production of chimeric RSV involves modifying existing, native RSV genes, as is further described. Accordingly, this example also describes the utility of this process in the directed attenuation of RSV pathogenicity, resulting in production of a vaccine with
30 defined, engineered biological properties for use in humans.

The first step of the rescue process involving the entire RSV RNA genome requires synthesis of a full length copy of the 15 kilobase (Kb) genome of RSV strain A2. This is accomplished by splicing together subgenomic double strand
35 cDNAs (using standard procedures for genetic manipulation) ranging in size from 1 kb-3.5 kb, to form the complete genomic cDNA. Determination of the nucleotide sequence of the genomic

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cDNA allows identification of errors introduced during the assembly process; errors can be corrected by site directed mutagenesis, or by substitution of the error region with a piece of chemically synthesized double strand DNA. Following
5 assembly, the genomic cDNA is positioned adjacent to a transcriptional promoter (e.g., the T7 promoter) at one end and DNA sequence which allows transcriptional termination at the other end, e.g., a specific endonuclease or a ribozyme, to allow synthesis of a plus or minus sense RNA copy of the
10 complete virus genome *in vitro* or in cultured cells. The leader or trailer sequences may contain additional sequences as desired, such as flanking ribozyme and tandem T7 transcriptional terminators. The ribozyme can be a hepatitis delta virus ribozyme or a hammerhead ribozyme and functions to
15 yield an exact 3' end free of non-viral nucleotides.

In accordance with this aspect of the invention, mutations, substitutions or deletions can be made to the native RSV genomic sequence which results in an increase in RSV promoter activity. Applicants have demonstrated that even
20 an increase in RSV promoter activity greatly enhances the efficiency of rescue of RSV, allowing for the rescue of infectious RSV particles from a full-length RSV cDNA carrying the mutation. In particular, a point mutation at position 4 of the genome (C to G) results in a several fold increase in
25 promoter activity and the rescue of infectious viral particles from a full-length RSV cDNA clone carrying the mutation.

The rescue process utilizes the interaction of full-length RSV strain A2 genome RNA, which is transcribed from the constructed cDNA, with helper RSV subgroup B virus proteins
30 inside cultured cells. This can be accomplished in a number of ways. For example, full-length virus genomic RNA from RSV strain A2 can be transcribed *in vitro* and transfected into RSV strain B9320 infected cells, such as 293 cells using standard transfection protocols. In addition, *in vitro* transcribed
35 genomic RNA from RSV strain A2 can be transfected into a cell line expressing the essential RSV strain A2 proteins (in the absence of helper virus) from stably integrated virus genes.

regulatory sequences within the virus genome), or minor (e.g., single or multiple nucleotide substitution(s), addition(s) and/or deletion(s) in key regulatory or functional domains within the virus genome), as further described in detail.

5 In addition to alteration(s) (including alteration resulting from translocation) of the RSV genetic material to provide heterologous sequence, this process permits the insertion of 'foreign' genes (i.e., genes non-native to RSV) or genetic components thereof exhibiting biological function
10 or antigenicity in such a way as to give expression of these genetic elements; in this way the modified, chimeric RSV can act as an expression system for other heterologous proteins or genetic elements, such as ribozymes, anti-sense RNA, specific oligoribonucleotides, with prophylactic or therapeutic
15 potential, or other viral proteins for vaccine purposes.

6.1. RESCUE OF THE LEADER AND TRAILER SEQUENCES OF RSV STRAIN A2 USING RSV STRAIN B9320 AS HELPER VIRUS

6.1.1.VIRUSES AND CELLS

20 Although RSV strain A2 and RSV strain B9320 were used in
this Example, they are exemplary. It is within the skill in
the art to use other strains of RSV subgroup A and RSV
subgroup B viruses in accordance with the teachings of this
Example. Methods which employ such other strains are
25 encompassed by the invention.

RSV strain A2 and RSV strain B9320 were grown in Hep-2 cells and Vero cells respectively, and 293 cells were used as host during transfection/rescue experiments. All three cell lines were obtained from the ATCC (Rockville, Maryland).

6.1.2. CONSTRUCTION & FUNCTIONAL ANALYSIS OF REPORTER PLASMIDS

Plasmid pRSVA2CAT (Fig. 1) was constructed as described below.

35 The cDNAs of the 44 nucleotide leader and 155 nucleotide trailer components of RSV strain A2 (see Mink et al., Virology

185:615-624 (1991); Collins et al., Proc. Natl. Acad. Sci. 88:9663-9667 (1991)), the trailer component also including the promoter consensus sequence of bacteriophage T7 polymerase, were separately assembled by controlled annealing of
 5 oligonucleotides with partial overlapping complementarity (see Fig. 1). The oligonucleotides used in the annealing were synthesized on an Applied Biosystems DNA synthesizer (Foster City, CA). The separate oligonucleotides and their relative positions in the leader and trailer sequences are indicated in
 10 Fig. 1. The oligonucleotides used to construct the leader were:

1. 5'CGA CGC ATA TTA CGC GAA AAA ATG CGT ACA ACA AAC TTG CAT AAA C
- 15 2. 5'CAA AAA AAT GGG GCA AAT AAG AAT TTG ATA AGT ACC ACT TAA ATT TAA CT
3. 5'CTA GAG TTA AAT TTA AGT GGT ACT
4. 5'TAT CAA ATT CTT ATT TGC CCC ATT TTT TTG GTT TAT GCA AGT TTG TTG TA
5. 5'CGC ATT TTT TCG CGT AAT ATG CGT CGG TAC

20 The oligonucleotides used to construct the trailer were:

1. 5'GTA TTC AAT TAT AGT TAT TAA AAA TTA AAA ATC ATA TAA TTT TTT AAA TA
2. 5'ACT TTT AGT GAA CTA ATC CTA AAG TTA TCA TTT TAA TCT TGG AGG AAT AA
- 25 3. 5'ATT TAA ACC CTA ATC TAA TTG GTT TAT ATG TGT ATT AAC TAA ATT ACG AG
4. 5'ATA TTA GTT TTT GAC ACT TTT TTT CTC GTT ATA GTG AGT CGT ATT A
5. 5'AGC TTA ATA CGA CTC ACT ATA ACG A
- 30 6. 5'GAA AAA AAG TGT CAA AAA CTA ATA TCT CGT AAT TTA GTT AAT ACA CAT AT
7. 5'AAA CCA ATT AGA TTA GGG TTT AAA TTT ATT CCT CCA AGA TTA AAA TGA TA
8. 5'ACT TTA GGA TTA GTT CAC TAA AAG TTA TTT AAA AAA TTA TAT GAT TTT TA
- 35 9. 5'ATT TTT AAT AAC TAT AAT TGA ATA CTG CA

transcription of a subgroup A RSV strain A2 RNA has to our knowledge hitherto not been formally reported.

6.2. CONSTRUCTION OF A cDNA REPRESENTING THE COMPLETE GENOME OF RSV

5 To obtain a template for cDNA synthesis, RSV genomic RNA, comprising 15,222 nucleotides, was purified from infected Hep-2 cells according to the method described by Ward et al., J. Gen. Virol. 64:167-1876 (1983). Based on the published
10 nucleotide sequence of RSV, oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer (Applied Biosystems, Foster City, CA) to act as primers for first and second strand cDNA synthesis from the genomic RNA template. The nucleotide sequences and the relative positions of the
15 cDNA primers and key endonuclease sites within the RSV genome are indicated in Fig. 3. The production of cDNAs from virus genomic RNA was carried out according to the reverse transcription/polymerase chain reaction (RT/PCR) protocol of Perkin Elmer Corporation, Norwalk, Connecticut (see also Wang et al., (1989) Proc. Natl. Acad. Sci. 86:9717-9721); the
20 amplified cDNAs were purified by electroelution of the appropriate DNA band from agarose gels. Purified DNA was ligated directly into the pCRII plasmid vector (Invitrogen Corp. San Diego), and transformed into either 'One Shot *E. coli* cells (Invitrogen) or 'SURE' *E. coli* cells (Stratagene, San Diego). The resulting, cloned, virus specific, cDNAs were assembled by standard cloning techniques (Sambrook et al., Molecular Cloning - A Laboratory Manual, Cold Spring Harbor laboratory Press (Cold Spring Harbor, NY, 1989) to produce a
25 cDNA spanning the complete RSV genome. The entire cDNA genome was sequenced, and incorrect sequences were replaced by either site-directed mutagenesis or chemically synthesized DNA. Nucleotide substitutions were introduced at bases 7291 and 7294 (with base number 1 being at the start of the genomic RNA
30 3' end) in the 'F' gene, to produce a novel *Stu* I endonuclease site, and at positions 7423, 7424, and 7425 (also in the F gene) to produce a novel *Pme* I site. These changes were
35

designed to act as definitive markers for rescue events. The bacteriophage T7 polymerase and the Hga I endonuclease site were placed at opposite ends of the virus genome cDNA such that either negative or positive sense virus genome RNA can be synthesized *in vitro*. The cDNAs representing the T7 polymerase promoter sequence and the recognition sequence for Hga I were synthesized on an Applied Biosystems DNA synthesizer and were separately ligated to the ends of the virus genome cDNA, or were added as an integral part of PCR primers during amplification of the terminal portion of the genome cDNA, where appropriate; the latter procedure was used when suitable endonuclease sites near the genome cDNA termini were absent, preventing direct ligation of chemically synthesized T7 promoter/Hga I site cDNA to the genome cDNA. This complete construct (genome cDNA and flanking T7 promoter/Hga I recognition sequence) was then cloned into the Kpn I/Not I sites of the Bluescript II SK phagemid (Stratagene, San Diego) from which the endogenous T7 promoter has been removed by site-directed mutagenesis. RNA transcribed from this complete genome construct may be rescued using RSV subgroup B helper virus to give infectious RSV in accordance with Example 6.1. This basic rescue system for the complete native, i.e., 'wild-type' RSV A2 strain genomic RNA can be employed to introduce a variety of modifications into the cDNA copy of the genome resulting in the introduction of heterologous sequences into the genome. Such changes can be designed to reduce viral pathogenicity without restricting virus replication to a point where rescue becomes impossible or where virus gene expression is insufficient to stimulate adequate immunity.

The following oligonucleotides were used to construct the ribozyme/T7 terminator sequence:

5' GGT*GGCCGGCATGGTCCCAGC
3' CCA CCGGCCGTACCAGGGTCG

35 CTCGCTGGCGCCGGCTGGGCAACA
GAGCGACCGCGGCCGACCCGTGTG

TTCCGAGGGGACCGTCCCCTCGGT

6.3. RESCUE OF A cDNA REPRESENTING THE COMPLETE GENOME OF RSV

6.3.1. THE CONSTRUCTION AND FUNCTIONAL ANALYSIS OF EXPRESSION PLASMIDS

5 The RSV, N, P, and L genes encode the viral polymerase of
RSV. The function of the RSV M genes is unknown. The ability
of RSV, N, P, M, and L expression plasmids to serve the
function of helper RSV strain AZ proteins was assessed as
described below. The RSV, N, P, L, and M2-1 genes were cloned
10 into the modified PCITE 2a(+) vector (Novagen, Madison, WI)
under the control of the T7 promoter and flanked by a T7
terminator at it's 3' end. PCITE-2a(+) was modified by
insertion of a T7 terminator sequence from PCITE-3a(+) into
the Alwn I and Bgl II sites of pCITE-2a(+). The functionality
15 of the N, P, and L expression plasmids was determined by their
ability to replicate the transfected pRSVA2CAT. At
approximately 80% confluency, Hep-2 cells in six-well plates
were infected with MVA at a moi of 5. After 1 hour, the
infected cells were transfected with pRSVA2CAT (0.5mg), and
20 plasmids encoding the N (0.4mg), P (0.4mg), and L (0.2mg)
genes using lipofectACE (Life Technologies, Gaithersburg,
M.D.). The transfection proceeded for 5 hours or overnight and
then the transfection medium was replaced with fresh MEM
containing 2% (fetal bovine serum) FBS. Two days post-
25 infection, the cells were lysed and the lysates were analyzed
for CAT activity using Boehringer Mannheim's CAT ELISA kit.
CAT activity was detected in cells that had been transfected
with N, P, and L plasmids together with PRSVAZCAT. However,
no CAT activity was detected when any one of the expression
30 plasmids was omitted. Furthermore, co-transfection of RSV-
GFP-CAT with the N, P, and L expression plasmids resulted in
expression of both GFP and CAT proteins. The ratios of
different expression plasmids and moi of the recombinant
vaccina virus were optimized in the reporter gene expression
35 system.

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6.3.2. RECOVERY OF INFECTIOUS RSV FROM THE COMPLETE RSV cDNA

Hep-2 cells were infected with MVA (recombinant vaccinia virus expressing T7 polymerase) at an moi of one. Fifty minutes later, transfection mixture was added onto the cells. The transfection mixture consisted of 2 μ g of N expression vector, 2 μ g of P expression vector, 1 μ g of L expression vector, 1.25 μ g of M2/ORF1 expression vector, 2 μ g of RSV genome clone with enhanced promoter, 50 μ l of LipofectACE (Life Technologies, Gaithersburg, M.D.) and 1 ml OPTI-MEM. One day later, the transfection mixture was replaced by MEM containing 2% FCS. The cells were incubated at 37°C for 2 days. The transfection supernatant was harvested and used to infect fresh Hep-2 cells in the presence of 40 μ g/ml aracycline (drug against vaccinia virus). The infected Hep2 cells were incubated for 7 days. After harvesting the P1 supernatant, cells were used for immunostaining using antibodies directed against F protein of RSV A2 strain. Six positively stained loci with visible cell-cell-fusion (typical for RSV infection) were identified. The RNA was extracted from P1 supernatant, and used as template for RT-PCR analysis. PCR products corresponding to F and M2 regions were generated. both products contained the introduced markers. In control, PCR products derived from natural RSV virus lacked the markers.

A point mutation was created at position 4 of the leader sequence of the RSV genome clone (C residue to G) and this genome clone was designated pRSVC4GLwt. This clone has been shown in a reporter gene context to increase the promoter activity by several fold compared to wild-type. After introduction of this mutation into the full-length genome, infectious virus was rescued from the cDNA clone. The rescued recombinant RSV virus formed smaller plaques than the wild-type RSV virus (Figure 8).

This system allows the rescue mutated RSV. Therefore, it may be an excellent tool to engineer live-attenuated vaccines against RSV and to use RSV vector and viruses to achieve heterologous gene expression. It may be possible to express G

protein of type B RSV into the type A background, so the vaccine is capable of protect both type A and type B RSV infection. It may also be possible to achieve attenuation and temperature sensitive mutations into the RSV genome, by
5 changing the gene order or by site-directed mutagenesis of the L protein.

6.4. USE OF MONOCLONAL ANTIBODIES TO DIFFERENTIATE RESCUED VIRUS FROM HELPER VIRUS

10 In order to neutralize the RSV strain B9320 helper virus and facilitate identification of rescued A 2 strain RSV, monoclonal antibodies against RSV strain B9320 were made as follows.

15 Six BALB/c female mice were infected intranasally (i.n.) with 10^5 plaque forming units (p.f.u.) of RSV B9320, followed 5 weeks later by intraperitoneal (i.p.) inoculation with 10^6 - 10^7 pfu of RSV B9320 in a mixture containing 50% complete Freund's adjuvant. Two weeks after i.p. inoculation, a blood sample from each mouse was tested for the presence of RSV
20 specific antibody using a standard neutralization assay (Beeler and Coelingh, J. Virol. 63:2941-2950 (1988)). Mice producing the highest level of neutralizing antibody were then further boosted with 10^6 p.f.u. of RSV strain B9320 in phosphate buffered saline (PBS), injected intravenously at the
25 base of the tail. Three days later, the mice were sacrificed and their spleens collected as a source of monoclonal antibody producing B-cells. Splenocytes (including B-cells) were teased from the mouse spleen through incisions made in the spleen capsule into 5 ml of Dulbecco's Modified Eagle's Medium
30 (DME). Clumps of cells were allowed to settle out, and the remaining suspended cells were separately collected by centrifugation at 2000xg for 5 minutes at room temperature. These cell pellets were resuspended in 15 ml 0.83 (W/V) NH_4Cl , and allowed to stand for 5 minutes to lyse red blood cells.
35 Splenocytes were then collected by centrifugation as before through a 10 ml; cushion of fetal calf serum. The splenocytes were then rinsed in DME, repelleted and finally resuspended in

expression of the M2/ORF1 gene function. In the present analysis, Hep-2 cells which are susceptible to RSV replication, were co-transfected with plasmids encoding the 'N', 'P' and 'L' genes of the viral polymerase of RSV and the cDNA corresponding to the full-length antigenome of RSV, in the presence or absence of plasmid DNA encoding the M2/ORF1 gene, and the number of RSV infectious units were measured in order to determine whether or not the M2/ORF1 gene product was required to rescue infectious RSV particles.

10 The following plasmids were used in the experiments described below: a cDNA clone encoding the full-length antigenome of RSV strain A2, designated pRSVC4GLwt; and plasmids encoding the N, P, and L polymerase proteins, and plasmid encoding the M2/ORF1 elongation factor, each
15 downstream of a T7 RNA promoter, designated by the name of the viral protein encoded.

pRSVC4GLwt was transfected, together with plasmids encoding proteins N, P and L, into Hep-2 cells which had been pre-infected with a recombinant vaccinia virus expressing the T7 RNA polymerase (designated MVA). In another set of Hep-2 cells, pRSVC4GLwt was co-transfected with plasmids encoding the N, P and L polymerase proteins, and in addition a plasmid encoding the M2 function. Transfection and recovery of recombinant RSV were performed as follows: Hep-2 cells were split in six-well dishes (35mm per well) 5 hours or 24 hours prior to transfection. Each well contained approximately 1×10^6 cells which were grown in MEM (minimum essential medium) containing 10% FBS (fetal bovine serum). Monolayers of Hep-2 cells at 70% - 80% confluence were infected with MVA at a multiplicity of infection (moi) of 5 and incubated at 35°C for 60 minutes. The cells were then washed once with OPTI-MEM (Life Technologies) and the medium of each dish replaced with 1 ml of OPTI-MEM and 0.2 ml of the transfection mixture. The transfection mixture was prepared by mixing the four plasmids, pRSVC4GLwt, N, P and L plasmids in a final volume of 0.1 ml OPTI-MEM at amounts of 0.5 - 0.6 μ g of pRSVC4GLwt, 0.4 μ g of N plasmid, 0.4 μ g of P plasmid, and 0.2 μ g of L plasmid. A

infectious units of RSV observed. Thus, the results of these experiments clearly indicate that RSV can be rescued in the absence of the M2/ORF1 from cells transfected only with plasmids encoding the three polymerase proteins, N, P and L, and the cDNA encoding the full-length RSV antigenome. The rescue of true RS virions in the absence of M2/ORF1 was further indicated by the ability to passage the rescued recombinant RSV for up to six passages. Therefore, the production of RSV virions is not dependent on the expression of the M2/ORF1 gene, nor does the inclusion of the M2/ORF1 gene in the transfection assay increase the efficiency of true RSV rescue.

Table I. Production of infectious RSV through plasmid transfection is not dependent on expression of M2ORF1

Expt.	Production of infectious RSV (pfu from 0.5ml transfection supernatants)	
	+ M2 ORF1	-M2 ORF1
1.	6,10(8)	16,9(13)
2.	120,46,428(198)	100,122,105(109)
3.	160,180(170)	150,133(142)
4.	588,253,725(522)	300,1000,110(470)

Each experiment was done singly, in duplicates or triplicates. The average number of plaque forming units (pfu) from 0.5 ml transfected cell supernatants is shown in the brackets.

8. EXAMPLE: EXPRESSION OF RSV SUBGROUP B-G AND -F PROTEINS BY RSV A2 STRAIN

The following experiments were conducted to generate a chimeric RSV which expresses the antigenic polypeptides of more than one strain of RSV. Two main antigenic subgroups (A and B) of respiratory syncytial virus (RSV) cause human diseases. Glycoproteins F and G are the two major antigenic determinants of RSV. The F glycoproteins of subgroup A and B viruses are estimated to be 50% related, while the relationship of G glycoproteins is considerably less, about 1-5%. Infection of RSV subgroup A induces either partial or no resistance to replication of a subgroup B strain and vice

versa. Both subgroup A and subgroup B RSV virus vaccines are needed to protect from RSV infection.

The first approach described herein is to make an infectious chimeric RSV cDNA clone expressing subgroup B antigens by replacing the current infectious RSV A2 cDNA clone G and F region with subgroup B-G and -F genes. The chimeric RSV would be subgroup B antigenic specific. The second approach described herein is to insert subgroup B-G gene in the current A2 cDNA clone so that one virus would express both subgroup A and B specific antigens.

8.1. Substitution of A2 G and F by B9320 G and F genes

RSV subgroup B strain B9320 G and F genes were amplified from B9320 vRNA by RT/PCR and cloned into pCRII vector for sequence determination. BamH I site was created in the oligonucleotide primers used for RT/PCR in order to clone the G and F genes from B9320 strain into A2 antigenomic cDNA (Fig. 4A). A cDNA fragment which contained G and F genes from 4326 nt to 9387 nt of A2 strain was first subcloned into pUC19 (pUCR/H). Bgl II sites were created at positions of 4630 (SH/G intergenic junction) and 7554 (F/M2 intergenic junction), respectively by Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). B9320 G and F cDNA inserted in pCR.II vector was digested with BamH I restriction enzyme and then subcloned into Bgl II digested pUCR/H which had the A2 G and F genes removed. The cDNA clone with A2 G and F genes replaced by B9320 G and F was used to replace the Xho I to Msc I region of the full-length A2 antigenomic cDNA. The resulting antigenomic cDNA clone was termed pRSVB-GF and was used to transfect Hep-2 cells to generate infectious RSVB-GF virus.

Generation of chimeric RSVB-GF virus was as follows, pRSVB-GF was transfected, together with plasmids encoding proteins N, P, L and M2/ORF1, into Hep-2 cells which had been infected with MVA, a recombinant vaccinia virus which expresses the T7 RNA polymerase. Hep-2 cells were split a day before transfection in six-well dishes. Monolayers of Hep-2

cells at 60% - 70% confluence were infected with MVA at moi of 5 and incubated at 35°C for 60 min. The cells were then washed once with OPTI-MEM (Life Technologies, Gaithersburg, MD). Each dish was replaced with 1 ml of OPTI-MEM and added with 0.2 ml of transfection medium. The transfection medium was prepared by mixing five plasmids in a final volume of 0.1 ml of OPTI-MEM medium, namely 0.6 µg of RSV antigenome pRSVB-GF, 0.4 µg of N plasmid, 0.4 µg of P plasmid, 0.2 µg of L plasmid and 0.4 µg of M2/ORF1 plasmid. This was combined with 0.1 ml of OPTI-MEM containing 10 µl lipofectACE (Life Technologies, Gaithersburg, MD U.S.A.). After a 15 minute incubation at room temperature, the DNA/lipofectACE was added to the cells and the medium was replaced one day later by MEM containing 2% FBS. Cultures were further incubated at 35°C for 3 days and the supernatants harvested. Aliquots of culture supernatants (PO) were then used to infect fresh Hep-2 cells. After incubation for 6 days at 35°C, the supernatant was harvested and the cells were fixed and stained by an indirect horseradish peroxidase method using goat anti-RSV antibody (Biogenesis, Sandown, NH) followed by a rabbit anti-goat antibody linked to horseradish peroxidase. The virus infected cells were then detected by addition of substrate chromogen (DAKO, Carpinteria, CA, U.S.A.) according to the manufacturer's instructions. RSV-like plaques were detected in the cells which were infected with the supernatants from cells transfected with pRSVB-GF. The virus was further plaque purified twice and amplified in Hep-2 cells.

Recombinant RSVB-GF virus was characterized by RT/PCR using RSV subgroup B specific primers. Two independently
30 purified recombinant RSVB-GF virus isolates were extracted with an RNA extraction kit (Tel-Test, Friendswood, TX) and RNA was precipitated by isopropanol. Virion RNAs were annealed with a primer spanning the RSV region from nt 4468 to 4492 and incubated for 1 hr under standard RT conditions (10 μ l
35 reactions) using superscript reverse transcriptase (Life Technologies, Gaithersburg, MD). Aliquots of each reaction were subjected to PCR (30 cycles at 94°C for 30 s, 55°C for

to those of ordinary skill in the art. The cell monolayers were lysed by RIPA buffer and the polypeptides were immunoprecipitated with either polyclonal antiserum raised in goat against detergent disrupted RSV A2 virus (Fig. 7, lanes 1-4) or antiserum raised in mice against undisrupted B9320 virions (Fig. 7, lanes 5-8). The radio labeled immunoprecipitated polypeptides were electrophoresed on 10% polyacrylamide gels containing 0.1% SDS and detected by autoradiography. Anti-RSV A2 serum immunoprecipitated the major polypeptides of the RSV A2 strain, whereas anti-B9320 serum mainly reacted with RSV B9320 G protein and the conserved F protein of both A and B subgroups. As shown in Fig. 7, a protein which is identical to the A2-G protein (lane 3), was immunoprecipitated from the rRSVA2(B-G) infected cells (lane 4) by using an antiserum against RSV A2. The G protein of RSV B9320 strain was not recognized by the anti-A2 antiserum. A protein species, smaller than A2-G protein, was immunoprecipitated from both B9320 (lane 6) and rRSVA2(B-G) (lane 9) infected cells using the antiserum raised in mice against B9320 virions. This polypeptide was not present in the uninfected and RSV A2 infected cells and likely is to represent the G protein specific to the RSV B 9320 strain. Amino acid sequence comparison of both A2 and B9320 RSV G proteins indicated that two additional potential N-glycosylation sites (N-X-S/t) are present in the RSV A2G protein, which may contribute to slower migration of the A2 G protein under the conditions used. The F protein of RSV B9320 also migrated slightly faster than RSV A2 F protein. The P and M proteins also showed mobility differences between the two virus subtypes. The identity of the polypeptide near the top of the protein gel present in FSV B9320 and rRSVA2(B-G) infected cells is not known. Antisera raised in mice ot RSV B9320 virions poorly recognized the N, P and M proteins are compared to the goat antiserum raised against the RSV A2 strain. The data described above clearly indicate that chimeric rRSV A2(B-G) expresses both the RSV A2 and B9320 specific G proteins.

8.2.1 Replication of Recombinant RSV in Tissue Culture

Recombinant RS viruses were plaque purified three times and amplified in Hep-2 cells. Plaque assays were performed in Hep-2 cells in 12-well plates using an overlay of 1%

- 5 methylcellulose and 1 x L15 medium containing 2% fetal bovine serum (FBS). After incubation at 35°C for 6 days, the monolayers were fixed with methanol and plaques were identified by immunostaining. Plaque size and morphology of rRSV was very similar to that of wild-type A2 RSV (Fig. 8).
- 10 However, the plaques formed by rRSVC4G were smaller than rRSV and wild-type A2 virus. The only genetic difference between rRSV and rRSVC4 was a single nucleotide substitution in the RSV leader region. Therefore, the smaller plaque size of rRSV A2(B-G) was not distinguishable from that of rRSVC4G.
- 15 The growth curves of rRSV, rRSVC4G and rRSV A2 (B-G) were compared to that of the biologically derived wild-type A2 virus. Hep-2 cells were grown in T25 culture flasks and infected with rRSV, rRSVC4G, rRSVA2(B-G), or wild-type RSV A2 strain at a moi of 0.5. After 1 hour adsorption at 37°C, the
- 20 cells were washed three times with MEM containing 2% FBS and incubated at 37°C in 5% CO₂. At 4 hour intervals post-infection, 250 µl of the culture supernatant was collected, and stored at -70°C until virus titration. Each aliquot taken was replaced with an equal amount of fresh medium. The titer
- 25 of each virus was determined by plaque assay on Hep-2 cells and visualized by immunostaining (*vide supra*). As shown in Fig. 9, the growth kinetics of rRSV is very similar to that of wild-type A2 virus. Maximum virus titer for all the viruses were achieved between 48 hr to 72 hr. The virus titer of
- 30 rRSVC4G was about 2.4-fold (at 48 hr) and 6.6-fold (at 72 hr) lower than rRSV and wild-type A2 RSV. The poor growth of rRSVC4G may also be due to the single nucleotide change in the leader region. The chimeric rRSV A2(B-G) showed slower kinetics and lower peak titer (Fig. 9).

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- pCITE-Lmutant: encoding mutant RSV L gene, 0.2 μ g
- pRSVL mutant: full-length genomic RSV of the positive sense (antigenome) containing the same L-gene mutations as pCITE-L mutant, 0.6 μ g

DNA was introduced into cells by lipofectACE (Life Technologies) in OPTI-MEM. After five hours or overnight transfection, the transfection medium was removed and replaced
10 with 2% MEM. Following incubation at 35°C for three days, the media supernatants from the transfected cells were used to infect Vero cells. The virus was recovered from the infected Vero cells and the introduced mutations in the recovered recombinant viruses confirmed by sequencing of the RT/PCR DNA
15 derived from viral RNA.

Examples of the L gene mutants obtained by charged to alanine scanning mutagenesis are shown in the Table II. Mutants were assayed by determining the expression of CAT by pRSV/CAT minigenome following co-transfection of plasmids expressing N, P and either wild-type or mutant L. Cells were harvested and lysed 40 hours post-transfection after incubation at 33°C or 39°C. The CAT activity was monitored by CAT ELISA assay (Boehringer Mannheim). Each sample represents the average of duplicate transfections. The amount of CAT produced for each sample was determined from a linear standard curve.

From the above preliminary studies, different types of mutations have been found.

30 9.3.1. DETRIMENTAL MUTATIONS

Seven L protein mutants displayed a greater than 99% reduction in the amount of CAT produced compared to that of wild-type L protein. These mutations drastically reduced the activity of the RSV polymerase and are not expected to be

35 viable.

Table 1. Demographic characteristics of the study population	
Age (years)	65.0 ± 10.0
Gender	
Male	50 (50.0%)
Female	50 (50.0%)
Education (years)	12.0 ± 2.0
Marital status	
Married	40 (80.0%)
Single	10 (20.0%)
Occupation	
Retired	30 (60.0%)
Unemployed	20 (40.0%)
Income (USD/month)	1,200 ± 300
Health status	
Good	30 (60.0%)
Poor	20 (40.0%)
Comorbidities	
Hypertension	15 (30.0%)
Diabetes	10 (20.0%)
Cholesterol	12 (24.0%)
Arthritis	8 (16.0%)
Other	5 (10.0%)

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SIMILAR OR HIGHER THAN WILD TYPE L PROTEIN

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10.1. M2-2 DELETION MUTANT

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RSV restriction fragment from 4478 to 8505. The RSV restriction fragment had been previously prepared by Quikchange site-directed mutagenesis (Stratagene, La Jolla, CA). Digestion of pET(S/B) with Hind III restriction enzyme removed a 234 nucleotide sequence which contained the majority of the M2-2 open reading frame. The nucleotides encoding the first 13 amino acids at the N-terminus of the M2-2 gene product were not removed because this sequence overlaps M2-1. The cDNA fragment which contained M2-2 gene deletion was digested with SacI and BamHI and cloned back into a full-length RSV cDNA clone, designated pRSVΔM2-2

Infectious RSV with this M2-2 deletion was generated by transfecting pRSVΔM2-2 plasmid into MVA-infected Hep-2 cells expressing N, P and L genes. Briefly, pRSVΔM2-2 was transfected, together with plasmids encoding proteins N, P and L, into Hep-2 cells which had been infected with a recombinant vaccinia virus (MVA) expressing the T7 RNA polymerase. Transfection and recovery of recombinant RSV was performed as follows. Hep-2 cells were split five hours or a day before the transfection in six-well dishes. Monolayers of Hep-2 cells at 70% - 80% confluence were infected with MVA at a multiplicity of infection (moi) of 5 and incubated at 35°C for 60 min. The cells were then washed once with OPTI-MEM (Life Technologies, Gaithersburg, M.D.). Each dish was replaced with 1 ml of OPTI-MEM and 0.2 ml transfection medium was added. The transfection medium was prepared by mixing 0.5 - 0.6 μg of RSV antigenome, 0.4 μg of N plasmid, 0.4 μg of P plasmid, and 0.2 μg of L plasmid in a final volume of 0.1 ml OPTI-MEM medium. This was combined with 0.1 ml of OPTI-MEM containing 10 μl lipofectACE (Life Technologies). After a 15 minute incubation at room temperature, the DNA/lipofectACE mixture was added to the cells. The medium was replaced one day later with MEM containing 2% FBS. Cultures were further incubated at 35°C for 3 days and the supernatants harvested. Three days post-transfection, 0.3 - 0.4 ml culture supernatants were passaged onto fresh Hep-2 cells and incubated with MEM containing 2% FBS. After incubation for

six days, the supernatant was harvested and the cells were fixed and stained by an indirect horseradish peroxidase method using goat anti-RSV antibody (Biogenesis) followed by a rabbit anti-goat antibody linked to horseradish peroxidase. The virus infected cells were then detected by addition of substrate chromogen (DAKO) according to the manufacturer's instructions. Recombinant RSV which contained M2-2 gene deletion was recovered from the transfected cells. Identification of rRSVΔM2-2 was performed by RT/PCR using primers flanking the deleted region. As shown in Fig. 12A, a cDNA fragment which is 234 nucleotides shorter than the wild-type RSV was detected in rRSVΔM2-2 infected cells. No cDNA was detected in the RT/PCR reaction which did not contain reverse transcriptase in the RT reaction. This indicated that the DNA product was derived from viral RNA and not from contamination. The properties of the M2-2 deletion RSV are currently being evaluated.

10.2. SH DELETION MUTANT.

To delete the SH gene from RSV, a Sac I restriction enzyme site was introduced at the gene start signal of SH gene at position of nt 4220. A unique SacI site also exists at the C-terminus of the SH gene. Site-directed mutagenesis was performed in subclone pET(A/S), which contains an AvrII(2129) SacI (4478) restriction fragment. Digestion of pET(A/S) mutant with SacI removed a 258 nucleotide fragment of the SH gene. Digestion of the pET(A/S) subclone containing the SH deletion was digested with Avr II and Sac I and the resulting restriction fragment was then cloned into a full-length RSV cDNA clone. The full-length cDNA clone containing the SH deletion was designated pRSVΔSH.

Generation of the pRSVΔSH mutant was performed as described above (see 10.1). SH-minus RSV (rRSVΔSH) was recovered from MVA-infected cells that had been co-transfected with pRSVΔSH together with N, P and L expression plasmids. Identification of the recovered rRSVΔSH was performed by RT/PCR using a pair of primers which flanked the SH gene. As

shown in Fig. 12A, a cDNA band which is about 258 nucleotides shorter than the wild-type virus was detected in the rRSVΔSH infected cells. No DNA was detected in the RT/PCR reaction which did not have reverse transcriptase in the RT reaction.

5 This indicated that the PCR DNA was derived from viral RNA and was not artifact, and that the virus obtained was truly SH-minus RSV.

10.3. GENERATION OF BOTH SH AND M2-2 DELETION MUTANT.

Both SH and M2-2 genes were deleted from the full-length RSV cDNA construct by cDNA subcloning. A Sac I to Bam HI fragment containing M2-2 deletion removed from cDNA subclone pET(S/B) Δ M2-2RSV was cloned into pRSV Δ SH cDNA clone. The double gene deletion plasmid pRSV Δ SH Δ M2-2 was confirmed by restriction enzyme mapping. As shown in Fig. 12B, the SH/M2-2 double deletion mutant is shorter than the wild-type pRSV cDNA.

Recovery of infectious RSV containing both the SH and M2-2 deletion was performed as described earlier. Infectious virus with both SH and M2-2 deleted was obtained from transfected Hep-2 cells.

Table II CAT Expression levels of Mutant RSV L-gene

25	Mut.	Conc. of CAT (ng/mL)		Charge Cluster	Charge to Alanine Change	Rescued Virus
		33°C	39°C			
30	A33	0.246	Bkg	5	135E, 136K	No
	A73	3.700	0.318	3	146D, 147E, 148 D	Yes
	A171	3.020	Bkg	3	157K, 158D	Yes
	A81	1.000	0.280	3	255H, 256K	Yes
	A185	Bkg	Bkg	3	348E, 349E	No
	A91	Bkg	Bkg	3	353R, 355R	No
35	A101	Bkg	Bkg	3	435D, 436E, 437R	No
	A192	1.960	Bkg	3	510E, 511R	Yes
	A11	0.452	Bkg	1	520R	Yes

	Mut.	Conc. of CAT (ng/mL)		Charge Cluster	Charge to Alanine Change	Rescued Virus
		33°C	39°C			
5	A111	2.320	0.267	4	568H, 569E	Yes
	A121	0.772	Bkg	2	587L, 588R	No
	A133	Bkg	Bkg	4	620E, 621R	No
	A141	2.800	Bkg	3	1025K, 1026D	Yes
10	A25	0.169	Bkg	3	1033D, 1034D	Yes
	A45	5.640	0.478	5	1187D, 1188K	Yes
	A153	4.080	0.254	5	1187D, 1188K, 1189R, 1190E	Yes
	A162	10.680	Bkg	3	1208E, 1209R	No
15	A201	Bkg	Bkg	3	1269E, 1270K	No
	A211	2.440	0.047	3	1306D, 1307E	Yes
	A221	0.321	Bkg	3	1378D, 1379E	No
	A231	Bkg	Bkg	3	1515E, 1516K	No
20	A241	1.800	0.308	3	1662H, 1663K	Yes
	A57	5.660	0.706	3	1725D, 1726K	Yes
	A65	3.560	0.168	2	1957R, 1958K	Yes
	A251	0.030	Bkg.	3	2043D, 2044K	Yes
25	A261	Bkg	Bkg	3	2102K, 2103H	No
	AD11	2.800	0.456	5 and 3	1187D, 1188K, 1725D, 1726K	No
	AD21	2.640	0.226	5 and 2	1187D, 1188K, 1957R, 1958K	No
	AD31	1.280	0.192	3 and 2	1725D, 1726K, 1957R, 1958K	No
30	F1	Bkg	Bkg	-	521 F to L	Yes
	F13	0.13	Bkg	-	521 F to L	Yes
	Lwt	3.16	-	-	no amino acid changes	Yes

- 63 -

single illustrations of individual aspects of the invention,
and any constructs, viruses or enzymes which are functionally
equivalent are within the scope of this invention. Indeed,
various modifications of the invention in addition to those
5 shown and described herein will become apparent to those
skilled in the art from the foregoing description and
accompanying drawings. Such modifications are intended to
fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of
10 which are incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

1. An isolated polynucleotide molecule comprising an operably linked transcriptional promoter, a polynucleotide sequence encoding a paramyxovirus genome and a transcriptional terminator wherein said polynucleotide sequence encoding said paramyxovirus genome is modified by introduction of a heterologous sequence.
2. The polynucleotide of Claim 1 wherein the paramyxovirus is respiratory syncytial virus.
3. The polynucleotide of Claim 1 wherein the paramyxovirus is parainfluenza virus.
4. A method for producing a recombinant paramyxovirus comprising:
 - a) providing to a host cell a polynucleotide encoding a paramyxoviral genome modified by the introduction of a mutation or a heterologous sequence, and polynucleotides encoding N, P and L proteins under conditions sufficient to permit the co-expression of the polynucleotides and the production of recombinant virus; and
 - b) isolating the virus produced by the host cell.
5. The method of Claim 4 wherein the virus is respiratory syncytial virus.
6. The method of Claim 4 wherein the virus is parainfluenza virus.

ABSTRACT

The present invention relates to genetically engineered recombinant RS viruses and viral vectors which contain heterologous genes which for the use as vaccines. In accordance with the present invention, the recombinant RS viral vectors and viruses are engineered to contain heterologous genes, including genes of other viruses, pathogens, cellular genes, tumor antigens, or to encode combinations of genes from different strains of RSV.

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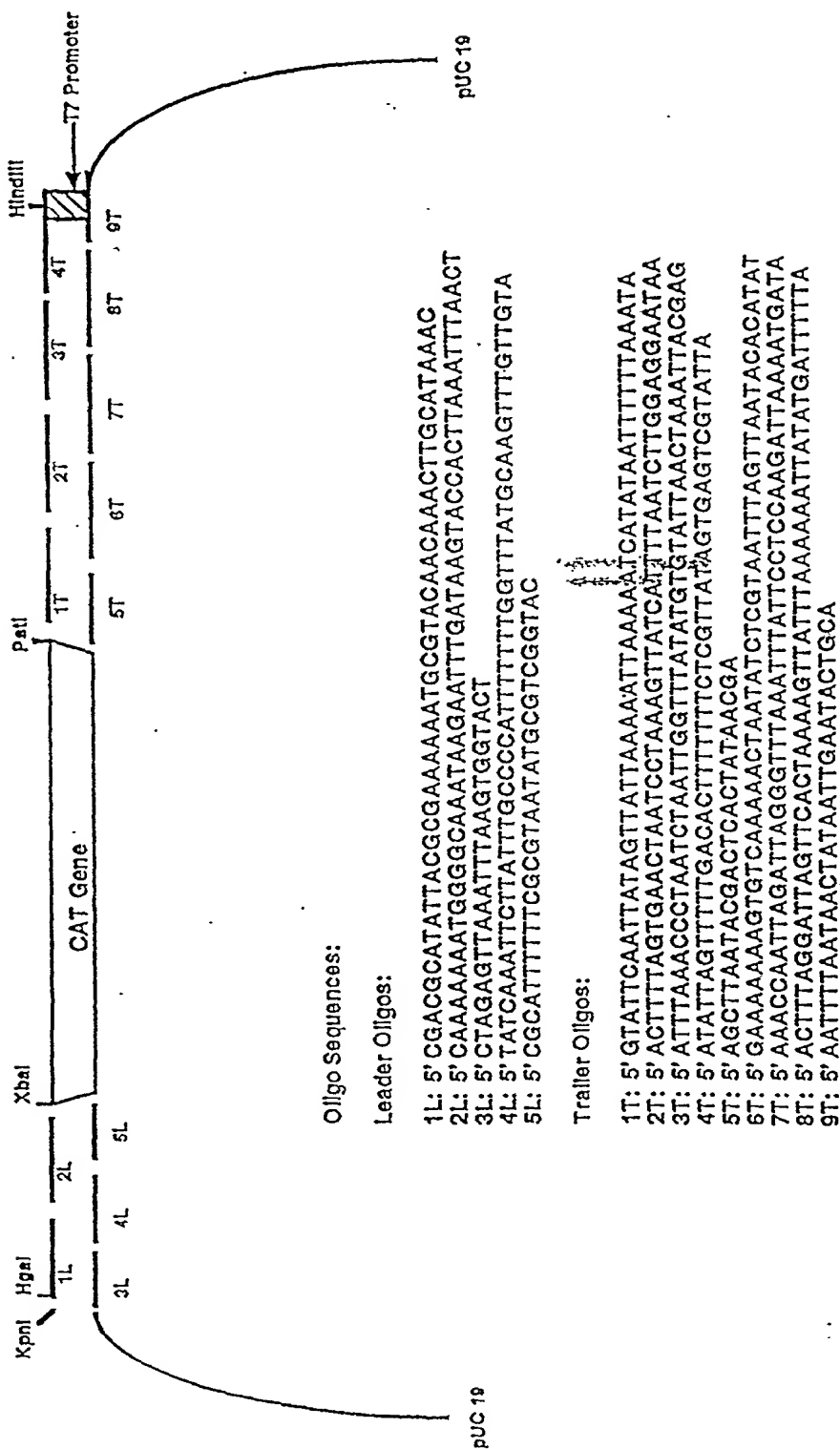


FIG. 1

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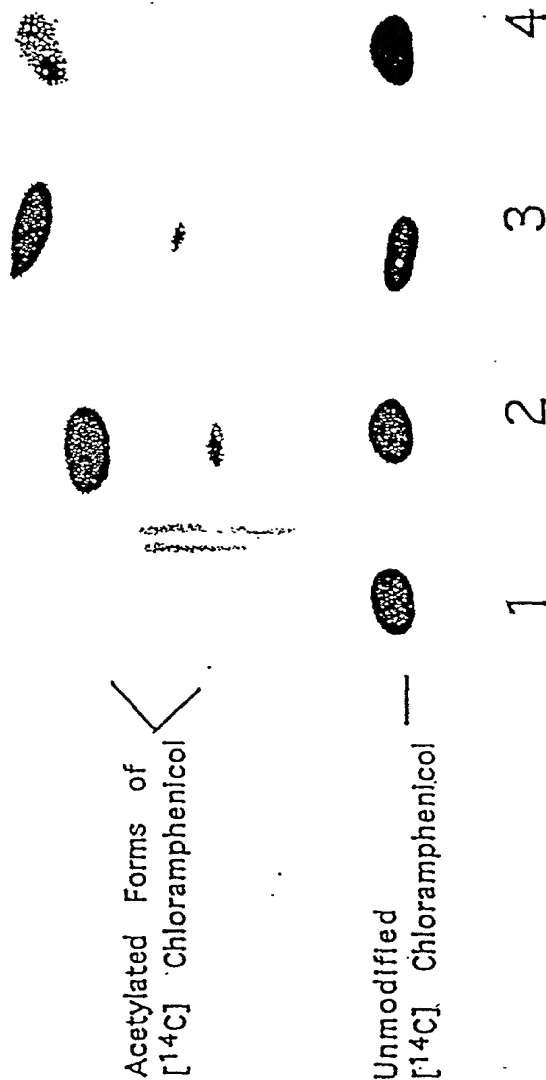
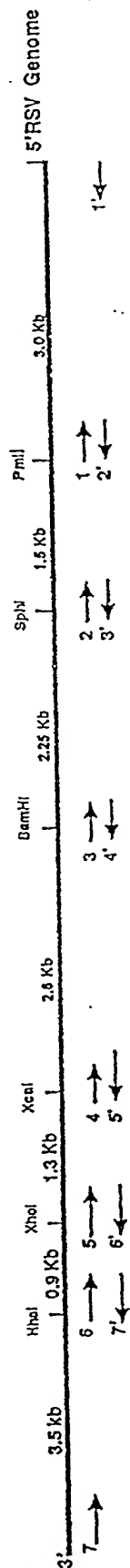


FIG. 2

008211 5/6/82/60



Primer Sequences:

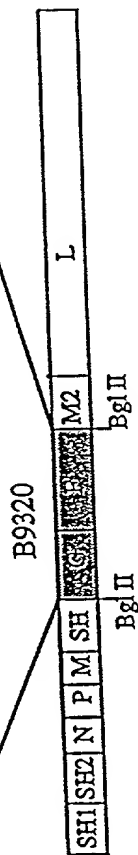
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 5: 5' TACCAAGAGCTCGAGTCA
 6: 5' TTTACCATATGCGCTAATGT
 7: 5' ACGGAAAAAATGCGTACA

1': 5' ACGAGAAAAAAGTGTCAC
 2': 5' CTCACCAAGTGTAAAC
 3': 5' GGTGCATGCCATATATGT
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 6': 5' TGAATCGAGCTCTTGGTA
 7': 5' ACATTAGCGCATATGGTAAA

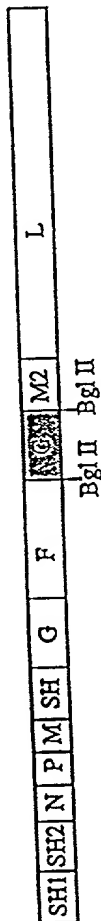
FIG. 3

A. RSVB-GF

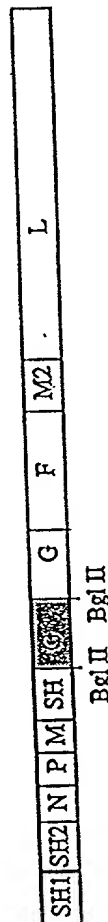
ATCAGGATCCACAATAACATTGGGGCAAAATGCAACC CAACTCATAGTTACATAAAACGGATCCGAATGCCAT
BamH I Gene start BamH I Gene stop



B. RSVB9320G-F/M2



C. RSVB9320G-SH/G



FIGS. 4A-C

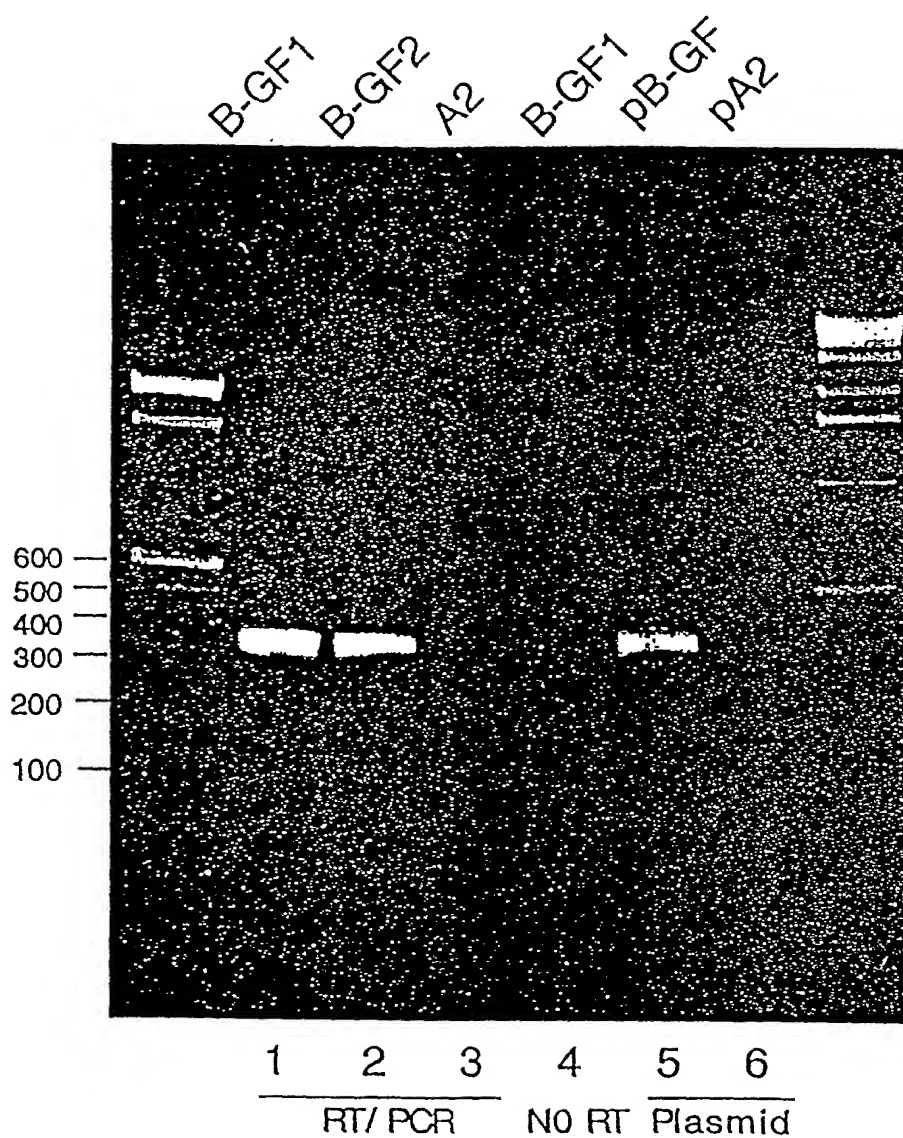
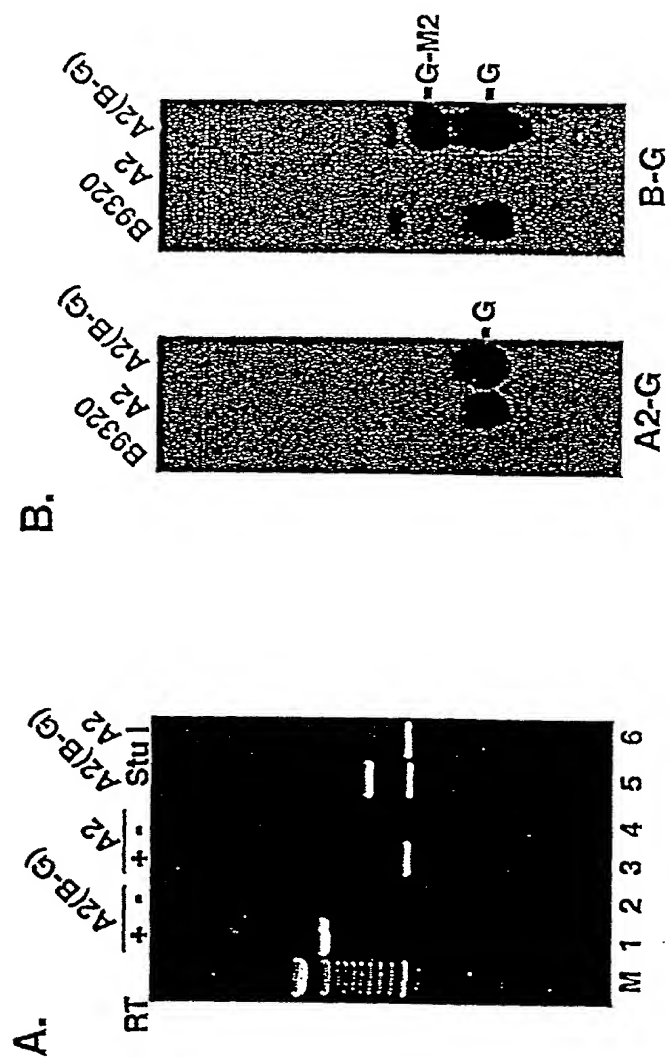


FIG. 5



FIGS. 6A-B

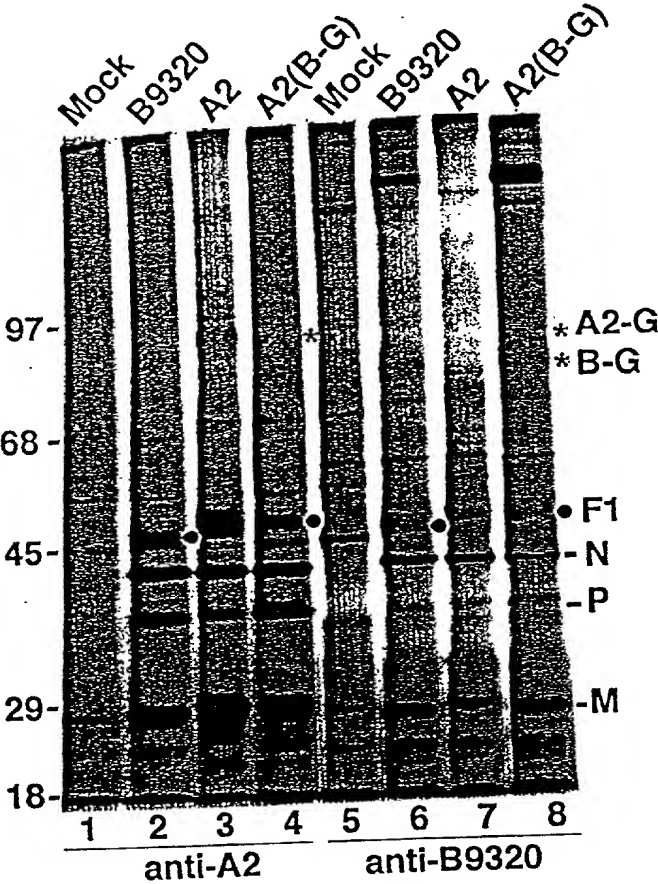


FIG. 7

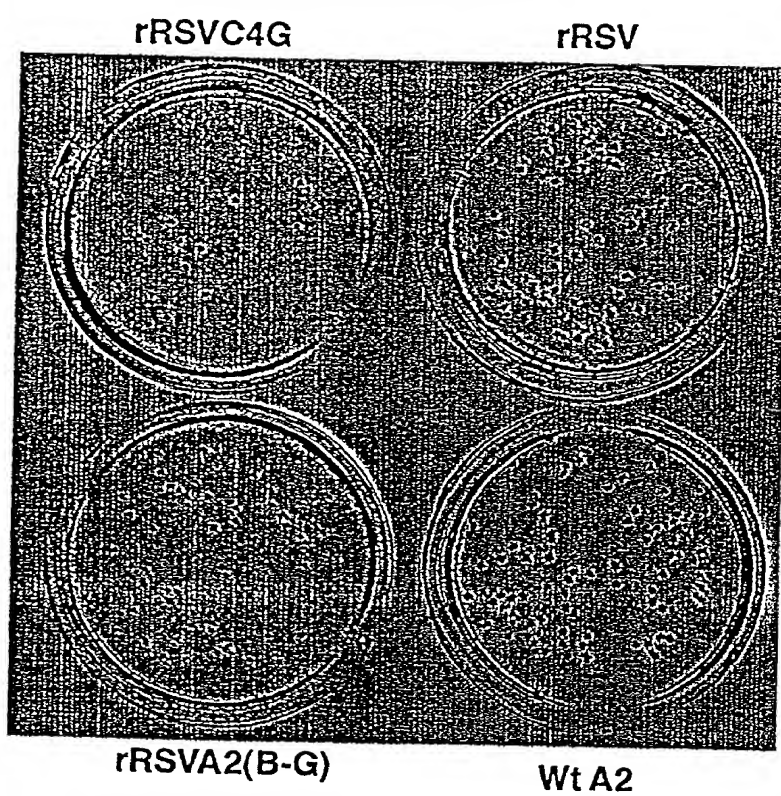


FIG. 8

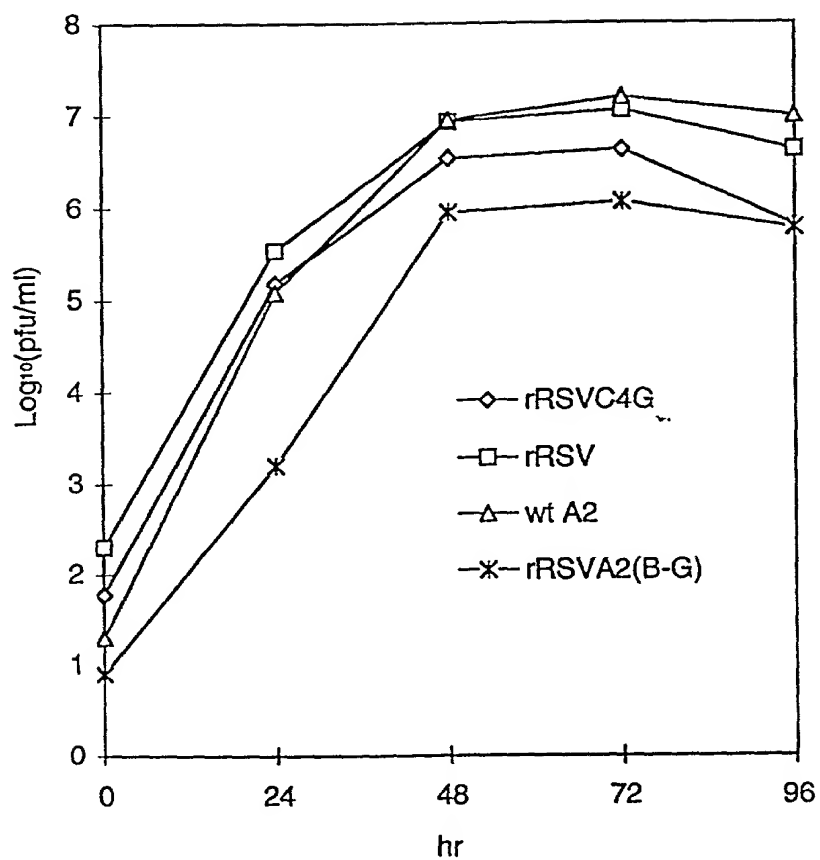


FIG. 9

MDPIINGSANVYLT DSYLKGVISFSECNA LGSYIFNGPYLKNDY TNLISRQNPLIEHMN LKKLNTQSLISKYH 75
 KGEIKLEPTFYQSL LMTYKSMTSSEQIAT TNLKKIIRRAIEIS DVKVYAILNKLGLKE KDKIKSNNGQDEEDNS 150
 VITTIKDDILSAVK DNQSHLKADKNHSTK QKDTIKTTLLKKLMC SMQHPSPWLHWFNL YTKLANNILTYQRSNE 225
 VKNHGFTLIDNQTLG GFQFILNQYGCIVYH KELKRIITVTTYNQFL TWKDISLSRLNVCLI TWISNCLNTINKSLG 300
 LRCGFNNVILTQLFL YGDCILKLFHNEGFI IITKEVEGFTMSLILN ITEEDQFRKEFYNSM LNNITDAANKAQKNL 375
 LSRVCHTLIDKTVSD NIINGRWIILLKSKFL KLIKLAGDNNLNLS ELYFLFRIFGHPMVD ERQAMDVAKINCNET 450
 KFYLLSSLMLRGAF IVRIIKGFVNNYNRW PTLRNAIVPLRWLT YYKLNTYPSLLELTE RDLIVLSGLRFYREF 525
 RLPKKVDLEMIINDK AISPKNLWTSFPR NYMPSHIQNYIEHEK LKFSESDKSRVLEY YLRDNKFNECDLYNC 600
 VVQSYLANPNHVS LTGKERELVSGRMFA MQPGMFRQVQILAEK MIAENILQFFPESLT RYGDLELQKILELKA 675
 GISNKSRYNDNINN YISKCSIIITDLSKEN QAFRYETSCICSDVL DELHGVQSLFSWLHL TIPHVTTICTYRHAP 750
 PYIGDHIVDLNNVDE QSGLYRYHMGGIEGW CQKLWITIEAISLIDL ISLKCKFSITALING DNQSIDISKPIRLME 825
 GQTHAQADYLLALNS LKLLYKEYAGIGHKL KGTETYISRDMQFMS KTIQHNQVYYPASIK KVLRVGFWINTILDD 900
 FKVSLESIGSLTQEL EYRGESLLCSLIFRN VMLYNQIALQKQHA LCNNKLYLDILKVLK HLKTFENLDNIDTAL 975
 TLYMNLPMFGGDP NLLYRSFYRRTPDFL TEAIVHSVFILSYT NHDLDKDLQDLSDDR LNKFLTCLITFDKNP 1050
 NAEFVTLMRDPQALG SERQAKITSEINRLA VTEVLSTAPNKFISK SAQHYTTTEIDLNDI MQNIEPTYPHGLRV 1125
 YESLPFYKAEKIVNL ISGTSITNILEKTS AIDLTIDRATENMR KNITLLIRILPLDCN RDKREILSMENLSIT 1200
 ELSKYVREBSWSLSN IVGVTSPSIMVTMDI KYTSTISSGIIIEK YNVNSLTRGERGPTK PWVGSSTQEKKTMPV 1275
 YNRQVLTQQDQID LLAKLDWVYASIDNK DEFMEELSIGTLGLT YEKAKLFPQYLSVN YLHRLTVSSRPFCEFP 1350
 ASIPAYRTTNYHFDI SPINRILTEKYGDED IDIVFQNCISFGLSL MSVVEQFTNVCNRI ILIPKLINEIHLMKPP 1425
 IFTGDVDIHLKQVI QKHMFPLPKISLTQ YVELFSLNKTLSGS HVNSNLLIAHKISDY FHNTYILSTNLAGHW 1500
 ILITQIMKDSKGIFE KDWGEGYITDHMFN LKVFENAYKTYLLCF HKGYGKAKLECDMNT SDLLCVLELIDSSYW 1575
 KSMKVFLEQKVIKY ILSQDASLHRVKGCH SFKLWFLKRLNVAEF TVCPWVUNIDYHPTH MKAILTYIDLVRMGL 1650
 TNIDRIHIKNHKKFN DEFYTSNLFYTNVF SDNTHLLTKHIRIAN SELENNYNKLYHPTP ETLLENILANPIKSND 1725
 KKTINDYCIGKNVDS IMLPLLNNKKLIKSS AMIRTNYSKQDLYNL FPMVVVIDRIIDHSGN TAKSNQLYTTTSHOI 1800
 SLVHNSSTLYCMLPW HHINRENFVFSSTGC KISIEYILKDLKID PNCIAFIFEGAGNLL LRTVVELHPDIRYIY 1875
 RSLKDCNDHSLPIEF LRLVNGHINIDYGEN LTIPATDATNNIHS YLHIKFAEPISLFVC DAELSVTVNWSKIII 1950
 EWSKHVRKCKYCSSLV NKCMLIVKYHAQDDI DFKLDNITILKTYVC LSKLKGSEVXLVLT IGPANIFFVFNVVQN 2025
 AKLILSRKNTFMPK KADKESIDANIKSLI PFCLCYPITKKGINPA LSKLKSVVSGDILSY SIAGRNEVFSNKLIN 2100
 HKHMMILKWFNHLVNL FRSTELNVNHLVMVE STYPYLSELINSLTT NELKKLIKITGSLLY NFHNE 2165

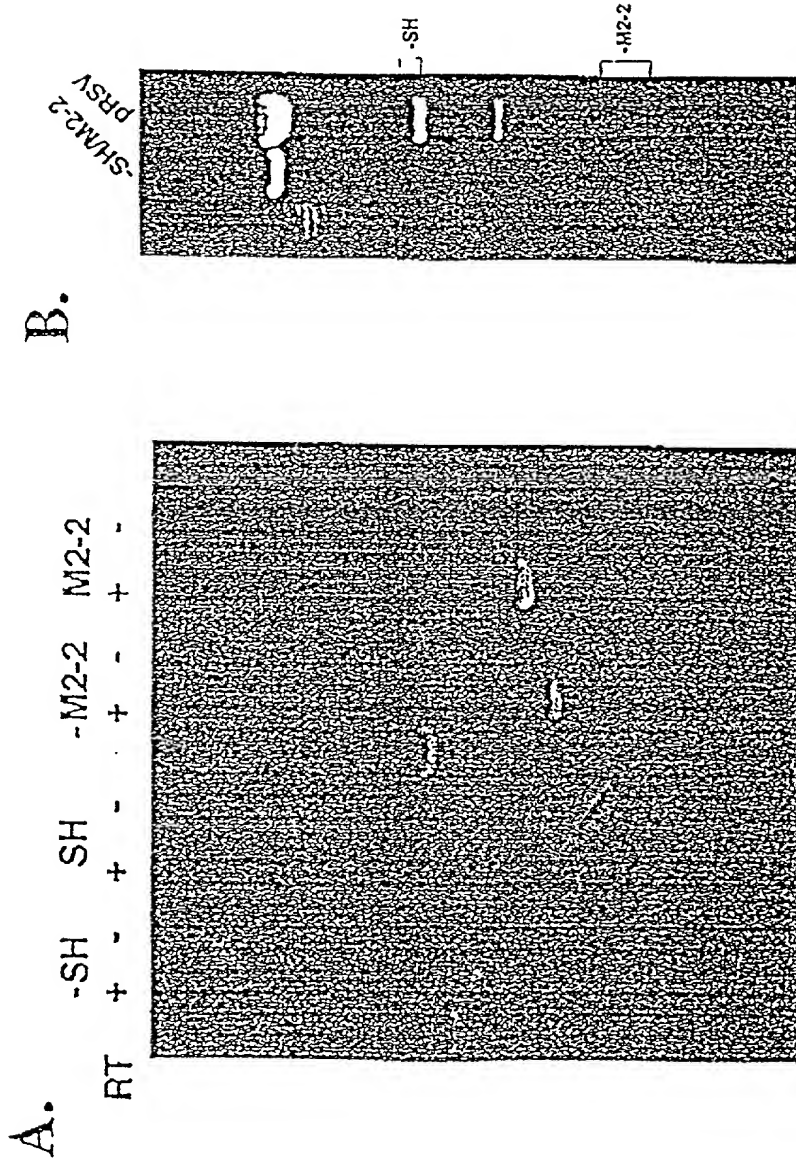
Charged Clusters (Amino Acids that are underlined were changed to alanines)
 Mutations in cpts-248/404
 Mutation in cpts530

FIG. 10

Cysteine residues
Cysteine residues that were changed to valine or aspartic acid
Cysteine residue deleted

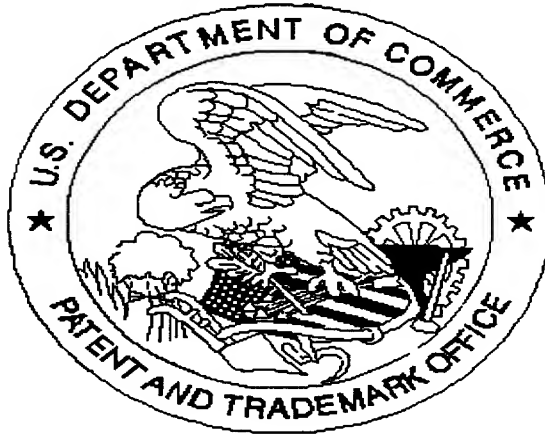
FIG. 11

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FIGS. 12A-B

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Application deficiencies were found during scanning:

☐ Page(s) _____ of _____ were not present
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☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

There are 66 sheets of specification.

☒ Scanned copy is best available. *drawings*

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